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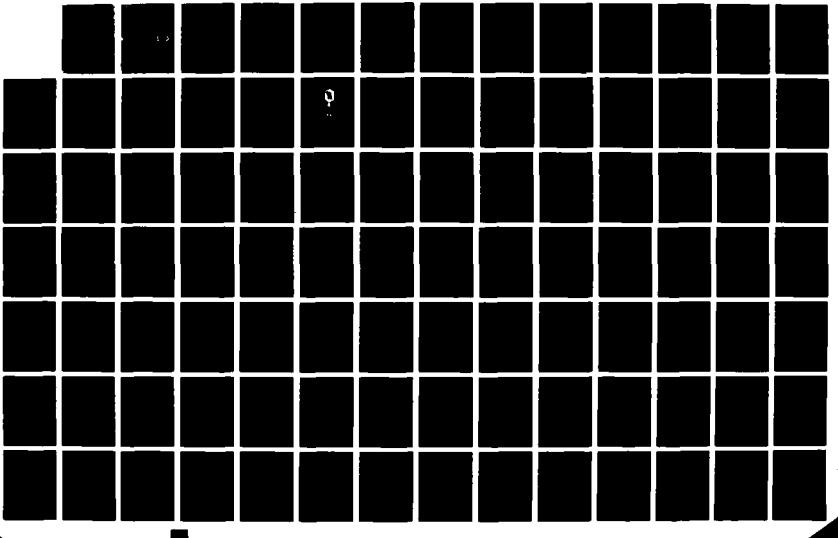

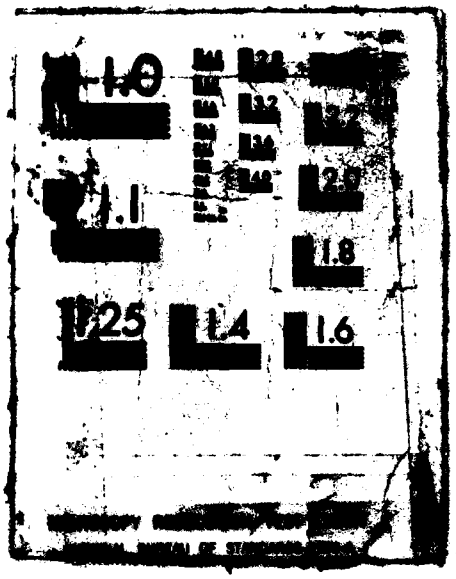
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ABSTRACT

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This region of T4 also encodes several other genes important in phage intra- and interspecific competition. They are B-gt, 42 and sp. Using recombinant DNA techniques, I precisely located the sp gene to a region between 21.647 and 22.014 kbp on the T4 restriction map and determined its molecular weight as approximately 15 kDa.

This same region of T4 was purported to contain gene 40. Complementation and marker rescue experiments with

sp+ plasmids indicated that genes sp and 40 are the same. Gene 40 mutants also were found to be defective in sp function. Genes sp and 40 were redesignated gene sp/40 thus linking an early expressing gene with the morphogenic pathway of prohead assembly.

Functionally, host enzymes exo III and exo V were found as participants in gp imm mediated exclusion. Presumably gp imm alters the pilot protein of the superinfecting DNA thus exposing it. Gp sp functions by an anti-lysozyme action. But the pleiotrophic effects of sp/40 are best explained by a temperature induced conformational rearrangement hypothesis.

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This work links molecular genetics to the ecological concept of competition and provides insights into the function and the evolutionary significance of the competition cluster genes. The competition cluster encodes fundamental adaptive strategies found universally in nature. theses; enzymes

GENETIC EXCLUSION IN BACTERIOPHAGE T4

by

John Wiiliam Obringer

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

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THE UNIVERSITY OF ARIZONA

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DEDICATION

Dedicated to Barbara, Aimée and Lisa Obringer.

From Burning JP-4 to the Midnight Oil,

Stick and Rudder to Testube and Quill,

From Slipping the Surly Bonds of Earth

to Exploring the Sultry Bonds of Life,

Ever Lofty

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This work links molecular genetics to the ecological concept of competition and provides insights into the function and the evolutionary significance of the competition cluster genes. The competition cluster encodes fundamental adaptive strategies found universally in nature.

CHAPTER 1

INTRODUCTION

T4 is a large bacteriophage of Escherichia coli containing a single linear double-stranded DNA molecule of about 166×10^3 base pairs. Its tightly organized genome encodes close to 200 genes which choreograph a complex, well regulated developmental process (reviewed in Guttman and Kutter, 1983). Bacteriophage T4, henceforth referred to as phage T4 or T4, consists of a virion approximately 215nm in total length and 80nm in width at the head. The capsid or head is an icosahedron made primarily of a protein layer attached to the connector vertex of the head. A neck structure containing a whiskered collar joins to the tail. The tail is 100nm long and composed of 20 different species of protein. It is the smallest contractile organ known to man. It consists of an outer sheath surrounding an inner tube through which the phage's DNA passes during the infection process. The distal portion of the tail is attached to a complex baseplate to which long and short tail fibers are fitted. The tail fibers are essential for infection by providing the primary host range determinants and effecting the adsorption process (Fig.1-1).

Phage T4 is morphologically classified as a urophage. It can be further classified as a member of the T-even-like phages along with its cousins T2 and T6 (Guttman and Kutter, 1983;see also Birge, 1981, for an overview). The T-even-like phages include a large number of types along as the TuI a and b, the TuII series, Ox2, 434, C21, etc. Many of them share serological as well as morphological characteristics (Schwartz, 1980; Schwartz et al., 1980). They also share a high degree of DNA homology (Kim and Davidson, 1974), and as a result recombine to varying degrees in mixed infections. Of all of the T-even-like phages, phage T4 has been the experimental workhorse. The past 50 years of experimentation have resulted in T4 being the most well understood genetic system in phage available for studying basic molecular phenomena including, as in the present case, genetic exclusion.

Genetic exclusion in phage T4 is the ability of a primarily infecting phage to prevent a secondarily infecting phage from contributing its genetic information to the progeny. Primarily infecting or primary phage is/are the first phage(s) to infect a cell; whereas, the secondarily infecting phage or secondary phage are those that infect after the primary phage(s) have begun its infectious cycle. Two or more genetically distinct T4

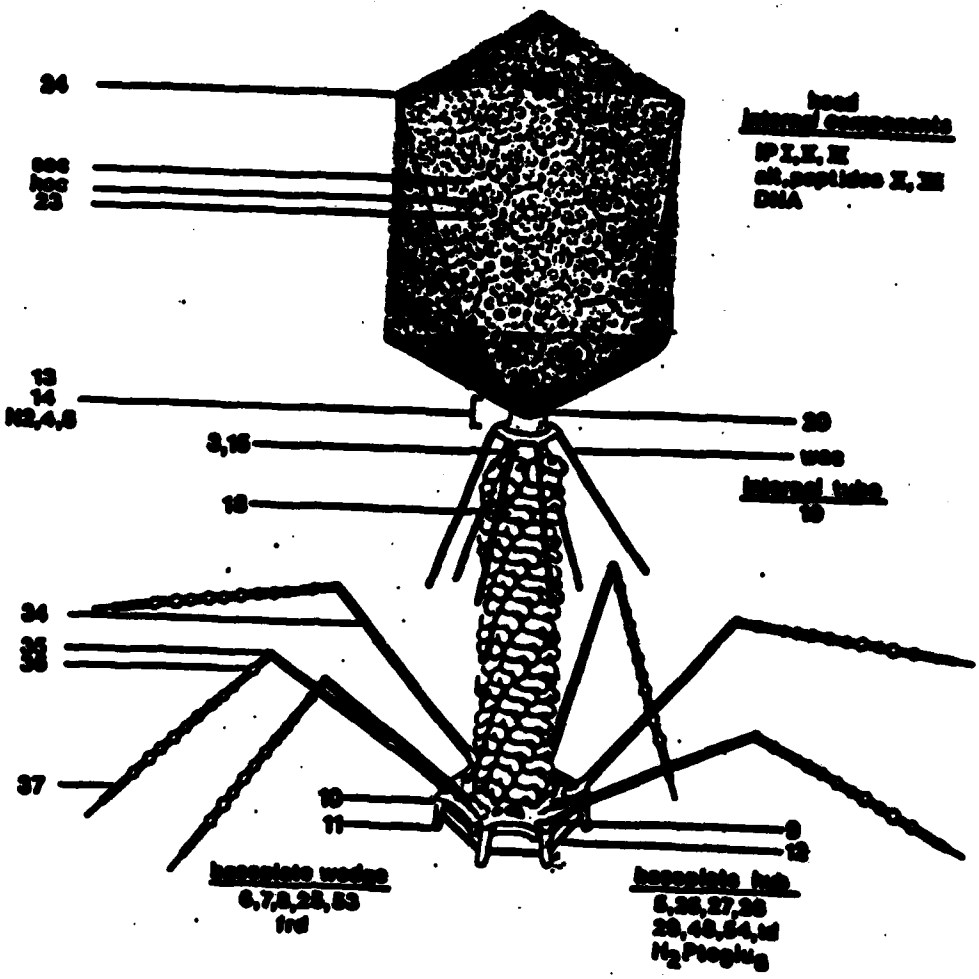


Fig. 1-1: Structure of bacteriophage T4

phage may contribute to the progeny if the infection is carried out simultaneously. Also if the infections are conducted sequentially in the presence of a host cell metabolic inhibitor, such as KCN, at sub-lethal concentrations both types of infecting phage will contribute to the progeny after the inhibitor is diluted out to innocuous levels (Cohen, 1949). However once the infectious cycle has begun, addition of secondarily infecting (or superinfecting) phage prompts the infected cell to respond in a number of characteristic ways, some of which lead to genetic exclusion.

Superinfection Phenomena

The superinfection of a host cell has been experimentally shown to elicit several responses. They are (i) lysis inhibition, (ii) lysis from without, (iii) superinfection breakdown and (iv) superinfection exclusion.

Lysis Inhibition

Lysis inhibition, first described by Doermann (1948), is characterized by a delay in lysis of the host cell as part of the normal phage life cycle. The normal time to lysis is 20-30 minutes but may be extended to several hours after superinfection. The process is not well understood.

Lysis From Without

Lysis from without (LFW) refers to the lysis of a host cell when infected with a high (>40 phage/cell) multiplicity of infection (moi) without phage production (Delbruck, 1940). However, when a cell is primarily infected and then superinfected with the same high moi as above the cell does not lyse (Visconti and Delbruck, 1953). Phage T4 infected cells become resistant to lysis from without through the expression of the spackle (sp) gene (Emrick, 1968). Several investigators (Cornett, 1974; Vallee and de Lapeyriere, 1975) have reported a resistance to LFW phenotype for both the imm (immunity) and sp genes with certain mechanistic differences; however, the results of Okamoto and Yutsudo (1974) indicate the imm is not involved.

Recently the probable mechanism of how the sp gene product prevents LFW was shown. Gene product (gp) 5, a component of the baseplate central plug (Kikuchi and King, 1975), was shown to have lysozyme activity and was proposed to be responsible for LFW (Nakagawa, Arisaka and Ischii, 1985). It was also shown by Kao and McClain (1980a) that the sp gp interacts with gp 5. They concluded that sp gp prevents LFW by interferring with the lysozyme activity of gp 5.

Superinfection Breakdown

Superinfection breakdown, originally described by Leslie et al. (1951) in phage T2, is the process by which the DNA of superinfecting phage is chemically degraded and appears in the host cell growth medium. Fielding and Lunt (1970) have shown that *E. coli* endonuclease I is the enzyme responsible for superinfection breakdown of T4. However, French et al. (1952), Hershey (1954) and Anderson and Eigner (1971) conclusively demonstrated that genetic exclusion occurs even in the absence of superinfection breakdown.

Genetic Exclusion

Genetic exclusion or superinfection exclusion, first noted by Delbruck and Luria (1942) as "interference" between various coliphages, was shown by Delbruck and Bailey (1946) to be the inability of superinfecting phage to contribute their genetic information to the progeny. The exclusion phenomenon in T-even phages was first reported by Dulbecco in 1952 and has been of interest to several investigators since then (Visconti, 1953; Fielding and Lunt, 1970; Anderson, Williamson and Eigner, 1971; Sauri and Earhart, 1971; Vallee, Cornett and Bernstein, 1972; Okamoto, 1973; Yutsudo and Okamoto, 1973). Despite the interest in the phenomenon among investigators, there has

been virtually no dicsussion of the adaptive benefit it has for the phage.

Bacteriophage Competition

The exclusion by primary phages of superinfecting phage genomes can be classified ecologically as competition - either interspecific (between species) or intraspecific (among individuals of the same species) (reviewed in Smith, 1966). Each type of competitive exclusion presumably bestows an advantage to the primary infecting phage. Interspecific exclusion allows the primary phage (predator) to sequester the resources of the host cell (prey) for the exclusive production of its own genome and incidentally that of its species. Additionally it is known that nonhomologous mixed infections (e.g. T2/T4, T2/T6, etc) result in lowered fitness among progeny (Mahmood and Lunt, 1972). Therefore, exclusion, by reducing nonhomologous mixed infections increases progeny fitness. [Fitness is defined as the survival value or reproductive capability of a given genotype as compared with another genotype in the population (Reiger, Michaelis and Green, 1976)]. With respect to intraspecific competition, exclusion may also act as an adaptive mechanism for promoting individual fitness since the protected resource is used for the production of the primary infecting phage's own unique

genome. In other words it is beneficial for the individual phage to be selfish.

Interspecific Competition in Phage

Adams (1959) first reported the production of only one type of progeny from a mixed infection of two unrelated phages. In mixed infections of more closely related phages a partial exclusion phenomenon is seen. Goldfarb and Kalinina (1970) observed about 90% exclusion of phage T2 by primary infecting phage T4. Phage T6 also has been shown to exclude phage T4 (Vallee and Cornett, 1972). These findings are further substantiated by others working in mixed infections among the T-even phages (Russell, 1974; Kim and Davidson, 1974; Okker, 1981; Okker, Pees and Bom, 1981). Similiar results have been obtained in the non T-even-type phages (Delbruck and Bailey, 1946; French et al., 1952; Benzer, 1955; Howard, 1967; Jacquemin-Sablon and Lanni, 1973; Pao and Speyer, 1975; Susskind and Botstein, 1979; Loewen, Miller and Warren, 1979; Toothman and Herskowitz, 1980b).

Intraspecfic Competition in Phage

Intraspecific competitive exclusion has been observed in numerous phages. T3 and T7 have a well developed homologous phage exclusion system that is triggered by a component of the viral particle (Hirsch-Kauffman et al., 1976). The early M gene of T7 has been shown to be

essential for the formation of the exclusion component (Schweiger et al., 1975). All of the T-even-like phages have been shown to express some degree of exclusion (Dulbecco, 1952; Anderson and Eigner, 1971). Phages lambda and P22 have an exclusion system based upon the repressors of the imm region (reviewed in Hendrix, 1983), and lambda has been shown to have an additional rex gene dependent exclusion component that acts on some nonhomologous as well as homologous phages (Toothman and Hershowitz, 1980 a, b and c). As a lytic phage, T4's strategy of superinfection exclusion differs considerably from those of temperate phages such as lambda and P22 (Birge, 1981). In T4 intraspecific exclusion (genetic exclusion) experimentally approaches 100% efficiency, although the mechanism has not been well defined. The two phage T4 genes shown to be primarily responsible for exclusion are imm and sp (Mufti, 1972; Childs, 1970; Childs 1973; Cornett, 1974; Vallee and de Lapyriere, 1975).

The Immunity Gene

The imm gene was named for its ability to provide "immunity" to superinfecting phage T4 and the disruptive effects of superinfecting T4 ghosts. It falls into a class of phage T4 genes known as the immediate early genes because they are transcribed by the host unmodified RNA polymerase and their expression begins immediately after

infection (Dulbecco, 1952; Peterson, Cohen and Ennis, 1972; O'Farrell and Gold, 1973; Yutsudo and Okamoto, 1973). Expression of the imm gene is blocked by drugs that inhibit the E. coli gyrase implying that the imm gene is under host gyrase control (Sinden and Pettijohn, 1982). The imm protein acts in a stoichiometric rather than in a catalytic manner, requires protein synthesis and its level of expression is independent of the multiplicity of infection (Vallee and Cornett, 1973). Although the synthesis of imm increases linearly for up to 10 minutes post infection (pi), it bestows nearly complete immunity by 3 minutes pi at 37°C. The imm gene mutants have been shown by various investigators including myself to be approximately 50% defective in the exclusion phenotype at 37°C, 4 minutes pi (Dulbecco, 1952; French et al., 1952; Sauri and Earhart, 1971; Cornett, 1974; Vallee and de Lapeyriere, 1975). By comparing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein gels of wildtype versus imm- phage, Yutsudo (1979) attributed two proteins to the imm gene (77 kilodaltons [kDa] and 45 kDa), while O'Farrell and Gold (1973) in a similiar manner assigned weights of 40 kDa and 28 kDa to the imm gene product(s). It is not clear why in both cases two proteins, rather than one, are altered in the imm- mutant and why the values from the two labs disagree. O'Farrell and Gold's molecular weight

determinations were corroborated by Sinden and Pettijohn (1982). The imm protein is postulated to act at the cell wall or membrane (Vallee and Cornett, 1973; Yutsudo and Okamoto, 1973), because superinfecting phage have a substantially reduced efficiency of DNA injection (Bayer, 1968; Cornett 1974). Most of the DNA that is injected by superinfecting phage is subsequently hydrolyzed by host endonuclease I found in the periplasmic space (French et al., 1951; Lesley et al., 1951; Hershey et al. 1954; Fielding and Lunt, 1970; Anderson and Eigner, 1971; Anderson et al., 1971; Yutsudo and Okamoto, 1973). The exact mechanism of action of the imm gp is still unknown.

Although the immunity function had been known since the late 1940's the responsible gene was not discovered until the early 1970's when two laboratories independently found and mapped a mutant defective in imm. Mufti (1972) discovered a mutant that was defective in immunity. Following up this finding in the same lab, Vallee and Cornett isolated and mapped a non-amber imm- mutant to a position between genes 42 (dCMP-hydroxymethylase) and 43 (DNA polymerase) using standard genetic recombinational mapping techniques (Vallee and Cornett, 1972; Cornett and Vallee, 1973). Independently, Childs (1973) using incomplete phage genomes confirmed this map position (Fig. 1-2, map position about 26.5 kilobase (kbp) from the border

of the rIIA-rIIB genes]. Cornett (1974) determined that his mutant and that of Childs' were isoallelic. Supporting the above map postion with molecular evidence, Jiang, Na and Xu (1984) and Lu et al. (1982) reported that a clone of the imm gene also contained a considerable portion of gene 42.

More recent information concerning the gene 43-imm-gene 42 region supports the previous work, but also poses a new dilemma. The 1985 "Bacteriophage Genomic Map" (Kutter and Ruger) places the carboxy end of gene 43 at approximately the 26.71 kb position and the amino terminal of gene 42 at approximately 26.2 kb. An immediate early promoter with the direction of transcription toward gene 42 has been mapped to the 26.3 kb region (Gram et al., 1984) (Fig. 1-2, 3-7). All of this taken together leaves about 0.5 kb between genes 42 and 43 to accommodate imm. Thus there is only room for an 18.5 kDa protein, which is only 24 to 66 percent of the information needed (the percentage depends on which size of the imm protein(s) measured on gels is correct).

Although the imm gene seems to account for the majority of the exclusion phenotype it is not the only gene involved in the process of genetic exclusion.

The Spackle Gene

The first spackle (sp) mutant was isolated by Emrick (1968) as a suppressor of an e mutant (lysozyme defective) in T4. She also found the sp mutant had reduced resistance to LFW, suggesting that sp gp is a phage-directed component of the bacterial cell wall. The name "spackle" was chosen to indicate spackling (or patching) of the host cell wall. Additionally she demonstrated that sp- infected cells are not subject to lysis inhibition upon superinfection, much like r- mutant phage infected cells (Doermann, 1948). Subsequently sp was determined to be an immediate early gene and was shown to account for approximately 20% of the wild type exclusion phenotype (Cornett, 1974; Vallee and de Laperiere, 1976; Peterson et al., 1982). Strangely, Cornett (1974) observed a reduced efficiency of DNA injection by superinfecting phage into cells primarily infected with sp- phage. He further speculated that the sp gp "affects the junctures of the cell wall and membrane with consequences similiar to those of the imm function." Okamoto and Yutsudo (1974) using a double imm- sp- T4 mutant deduced that the imm and sp gps probably act "cooperatively rather than additively" in the establishment of resistance to LFW by superinfecting phage and ghosts. Their results were substantiated by Vallee and de Lapeyriere (1975). [Ghosts are DNA-less particles produced

by osmotic shock (Duckworth, 1970, 1971). They can be used to experimentally distinguish the effects of the adsorption process alone from those of the overall injection process including DNA uptake.] Vallee and de Lapeyriere (1975) further imply that exclusion may have a phage encoded component that is not related to the imm or sp genes. Okamato (1973) also suggests that additional phage gene(s) are involved in immunity to ghosts. No other T-even-like phage has been shown to have mutants similiar to phage T4's sp mutant (Okamoto and Yutsudo, 1974). The only attempt to map the sp gene was done by Emrick (1968) who located it between genes 41 and 58/61 [about 20 kbp on the T4 map (Kutter and Ruger, 1985) (Fig. 1-2, 3-7)]. There is no molecular weight data on the sp gp.

Recent molecular insights partially clarify the mechanism of sp gp function. Gene product 5 was originally described as a component of the baseplate central plug (Kikuchi and King, 1975), but has recently been shown to have lysozyme activity (Loeb, 1974; Kao and McClain, 1980a; Nakagawa et al., 1985). Kao and McClain (1980a) speculated that gp 5 normally funtions in the "initiation of infection by catalyzing local cell wall digestion to facilitate penetration of the tail tube through the cell envelope." Nakagawa et al. (1985) later isolated and biochemically characterized gp 5 as a lysozyme. Kao and McClain (1980b)

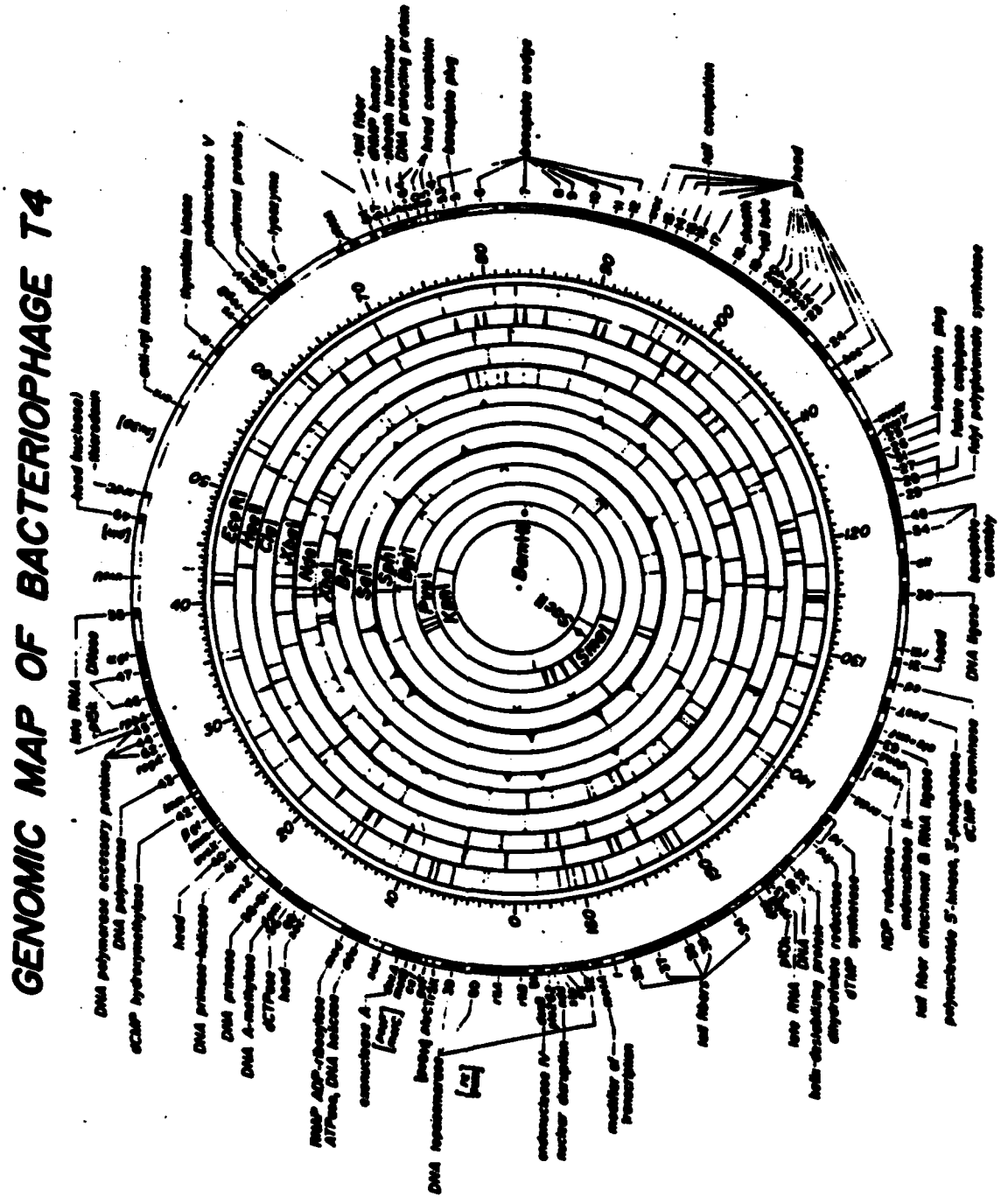


Fig 1-2: Genomic Map of Bacteriophage T4(taken from Kutter and Ruger, 1985).

had previously proposed an interaction between gps sp and 5 well as the reduced resistance to LFW of sp-. Apparently the interaction of gp sp with gp 5 prevents functional expression of the gp 5 lysozyme activity. That is, in the presence of gp sp, gp 5 (a lysozyme) cannot participate in lysis from within or LFW. Therefore, the wildtype gp sp bestows resistance to LFW and prevents premature cell lysis by wildtype phage by the gp 5 of the progeny. Given the above it would seem likely that gp sp inhibits gp 5 of secondarily absorbing phage to promote genetic exclusion.

Gp 25 is a component of the tail baseplate found in the outer wedges which surround the hub (Kikuchi and King, 1975). Like gp 5, gp 25 has recently been shown to have lysozyme activity (Szewczyk and Skorko, 1983; Szewczyk, Bienkowsky-Szewczyk and Kozloss, 1986). But unlike gp 5, gp 25 has not been directly shown to be involved in the initiation of infection or host cell lysis.

Phage T4 Infection

The infection process of phage T4 essentially allows the phage to inject its genome into a suitable *E. coli* host cell in order to permit a cycle of phage growth. Infection by phage T4 has been extensively studied (reviewed by Goldberg, 1983). Although T4 relies upon random movement (Brownian motion) to collide with host bacteria its ability to properly recognize and infect

metabolically suitable host cells is exquisite resulting in efficiencies of successful infection that approach 100%. This is a remarkable achievement compared to phages such as lambda, T7 and T5 whose efficiencies rarely exceed 25%, and to other phages which are even much less efficient. These reported efficiencies of infection are based upon absolute efficiencies of plating (EOP). (EOP is a quantitative expression of the number of phage that productively infect versus the total number possible.) In calculating the absolute EOP, the total number of phage particles per volume is estimated using electron microscopy and this is compared to the number of plaque forming particles per volume. The high efficiency of phage T4 infection has been attributed to the tail structure. After encountering a host cell the phage seeks out potential adsorption sites with its six long tail fibers. Their distal end interacts in a reversible manner with the lipopolysaccharide (LPS) (plus OmpC on E. coli K) on the host cell surface. When a suitable site is located, irreversible attachment to the cell surface takes place primarily via the baseplate fibrils (the short tail fibers). Bayer (1968, 1979) and Bayer, Costello and Bayer, (1982) have reported that at the phage T4 attachment site there are adhesion zones between the inner and outer membrane that possibly penetrate the cell wall. Others (Wright, McCann II and

Kanegasaski, 1980; Ishidate et al., 1986) have corroborated their observations. [Some investigators believe that imm gp mediated exclusion occurs at these sites (Childs, 1973; Vallee and de Lapeyriere, 1975) by preventing efficient adsorption. The imm gp may also act by preventing DNA ejection or uptake (Vallee and Cornett, 1972; Vallee and de Lapeyriere, 1975; Goldberg, 1980)]. Once irreversible attachment has occured, DNA injection is triggered by a molecular rearrangement of the baseplate. The tail sheath contracts allowing the tail tube to penetrate through the outer membrane and cell wall to the outer surface of the host cell inner membrane. The DNA is then extruded from the phage head through the hollow tail tube. Guided by a postulated pilot protein to a hypothetical uptake pore on the inner membrane the phage chromosome passes through the membrane into the cell's cytoplasm. Phage DNA uptake is energy dependent and appears to be regulated by a gating phenomenon. Indications are that the passage of the T1 DNA through the inner membrane is regulated by an electric potential across the membrane established by various ions. Inside the cell the phage chromosome occupies a proposed expression slot (Snustad, 1966, 1969) presumably on the inner leaf of the cytoplasmic membrane and begins gene expression and replication.

The lethal effect of ghost adsorption may be explained by the considerable amount of unrepaired membrane damage done to the host cell. Presumably a productive infection leads to gene expression which reverses or patches up the host cell membrane (Puck and Lee, 1955; Silver, Levine and Speilman, 1968) possibly through the action of gp sp and probably other gps. If all goes well during host cell take-over and T4 gene expression the various components of the progeny phage are constructed, the original DNA is multiply replicated, and then all of the components are assembled in a neatly organized particle.

Gene 40 and its Role in Head Morphogenesis

I will briefly review the role of gene 40 in head morphogenesis for reasons that will become clear later. Phage T4 head morphogenesis represents one of the most complex assembly processes thus far analyzed at the molecular level (reviewed by Black and Stowe, 1983). The icosohedral capsid is constructed largely of multiple units of a single major protein (gp 23). In the completed phage the capsid surrounds the genome, a single, linear DNA molecule. The head is manufactured by a series of sequential steps involving 13 known major structural components and numerous enzymatic and accessory proteins. The basic outline of events is as follows: (1) Prehead assembly: About 3000 protein molecules are assembled into

two or possibly three layers all originating from a common initiation complex which is anchored to the inner membrane of the host cell. (2) Prohead maturation: This involves the cleavage of a number of the prohead components to form a stable capsid which is released from the membrane. (3) DNA packaging: The free prohead is filled with DNA using additional structural and enzymatic proteins. (4) Shell transformation and capsomere maturation: This is marked by a striking change in prohead size and protein composition resulting in an antigenically distinct, more stable structure. (5) Head maturation: The final head and neck proteins are added giving the head the finishing touches needed to participate in tail attachment.

The first step in prohead assembly is prohead initiation. There are 3 major gene products involved- gp22, gp20 and gp40. Gp22 is a major core protein of the prohead and gp 20 is the proximal-vertex protein. Gp 20 can be detected on either the bacterial membrane of infected cells or in the prohead (Fig. 1-1). It is thought that gp 20 acts as a "workbench" anchored to the bacterial membrane upon which the prohead is assembled, except in this case the workbench is included in the product. Gp 20 has also been implicated in DNA packaging by acting at an initial step in phage DNA uptake (Hsiao and Black, 1977; Hsiao and Black, 1978c). Gp 40 is found only in

association with the bacterial membrane but is not found in the prohead. It is essential (required for phage production) only at high temperatures, but in its absence even at permissive temperatures phage production decreases by 80%. Hsiao and Black (1978a) have presented compelling genetic evidence that gp 40 specifically interacts with gp 20 during prohead assembly. They further proposed that gp 40 "helps provide a membrane-binding site for the proper assembly of (g)p 20 from which the assembly of T4 pre(pro)head on the membrane is initiated." Continuing, they (1978b) reported that gene 40 is located between genes 41 and 42 (Fig. 1-2, 3-7) [at about 22kbp on the T4 map (Kutter and Ruger, 1985)]. This map position was confirmed by others (Mattson et al., 1977; Hinton and Nossal, 1986). A striking feature of the map position of gene 40 is its location in an early region of the T4 genome away from the late regions that encode all of the other genes involved in capsid production. They further determined gene 40 to be under quasi-late regulation, producing a protein with a molecular weight of around 14 kDa. Other investigators have determined the molecular weight of gp 40 to be from 14 to 18 kDa (Castillo et al. 1977; Laemmli, 1970; Brown and Eiserling, 1979 a and b). The current thinking is that gp 40 modifies the bacterial inner membrane which facilitates a tight binding of gp 20,

the first structural protein in the head morphogenesis pathway (personal communication, Black, 1987). Other investigators have recently confirmed gp 20's essential role in capsid formation but question its function in core initiation. They additionally report that gp 20 contains two separate domains. The C-terminal domain is involved in the interaction with gp 23 whereas the N-terminal domain recognizes the core-membrane junction (Traub and Maeder, 1984; Kuhn et al., 1987). Presumably gp 40 interacts with the gp 20 N-terminal domain.

CHAPTER 2

MATERIALS AND METHODS

Media

Bacteria were grown in Hershey's broth (HB) or on enriched Hershey's agar plates. These were prepared as prescribed by Steinberg and Edgar (1962). When antibiotic media was required the appropriate amount of antibiotic, salts or stock solution, was added to the concentrations recommended in Maniatus, Fritsch and Sambrook (1982). M9 adsorption salts solution was used in diluting phage and for promoting adsorption of phage to host cells in various assays. To prepare this solution the following were added per liter of distilled deionized water: 5.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, and the pH adjusted to 6.8 to 7.0. All media were autoclaved, cooled to 55 ^{o}C to add non-autoclavable supplements, and then allowed to cool further to room temperature before use. Soft top agar (enriched Hershey's plate media with 1/2 the agar added) was used at a temperature of 45 ^{o}C where it is liquid. Indicator cells and phage were mixed in the soft agar before plating to titer phage.

Bacteria, phage and plasmid strains

The strains used, their relevant characteristics and the source or a reference are listed in Table 2-1.

Bacterial cultures

Bacterial cultures were stored for long term use in agar slants and stabs or by freezing at -70 ^{o}C in a mixture of 50% glycerol and 50% overnight culture. Overnight cultures were grown in HB at 37 ^{o}C from slant innocula weekly or as required. Experimental host bacteria were prepared by diluting overnight cultures 100-fold into the appropriate medium and incubating them at the experimental temperature with aeration. The bacterial densities were determined at frequent intervals using a Petroff-Hauser counting chamber. The bacteria were generally grown to 1-2 x10^{8} /ml and diluted to the desired concentration. Centrifugation of cells was generally conducted at 2,000 x g RCF. Plating indicators were prepared by growing the host cells to about 4 x 10^{8} /ml, concentrating 25-fold by centrifugation, and resuspending in fresh HB.

Titration of cells and phage plaque forming units

All titering of cell colony forming units (cfu) was carried out on enriched Hershey's agar plates with top agar overlays. Phage titers were determined after dilution by plaque formation on agar plates by the agar overlay method of Adams (1959). An aliquot of the phage-containing

Table 2-1: Bacteria, Plasmids and Phage Strains Used

Bacteria Strain	Genotype or comments	Source/ reference
E. coli		
S/6	su-	This lab
CR63	su+ (Su I, serine)	This lab
K803	su+	This lab
KP360	recBC, sbcB	K. Peterson (a)
DE828	594(recD1014)	D. Ennis (a)
594	su-(parent of DE828)	K. Peterson (a)
CES201	recBC, sbcB	CGSC (b)
JC5519	su+, recBC	CGSC
JC7623	su+, recBC, sbcB	CGSC
G240	su-	CGSC
G240R4	Su 9, UGA, trp	CGSC
U11R1d	Su 5, UAA-UAG, lysine	CGSC
H12R7a	Su 4, UAA-UAG, tyrosine	CGSC
DH5	Su 2, lacZdel, r-	M. Moran (a)
KL16	su-(parent of BW9101)	B. Wein (c)
BW9101	xth (Exo III-)	"
Plasmids		
pBR325	amp, tet, cam, 5996 bp	Prentki, 1981
pBB1	amp	This study
pPP1	tet	This study
pBHE3	amp	This study
pBBH	amp	This study
pBSK101	amp, tet	Fujisawa, 1985
pUC18	amp, expression vector	BRL (d)
pJO11	amp, T4 sp clone	This study
T4 Phage		
TID+	wildtype	This lab
am21x3	(42 amber)	J. Wiberg (e)
am269x3	"	"
amC87	"	"
amNG93	"	"
amE142, imm-	(39 amber, imm)	This lab
amNG372	(55 amber)	"
amNG205	(42 amber)	"
amNG205 sp-	(42 amber, sp)	"
amNG205 imm-, sp-	(42 amber, imm, sp)	"
amN93	(11 amber)	"
amE498	(42 amber)	B. Wood (f)
amN122	"	"
amNG411	"	"

amE117	"	"
amE774	"	"
amNG55x5	"	"
amNG554	(42 amber)	B. Wood
amCT36	"	"
amE645	"	"
amNG394	"	"
amNG352	"	"
amE385	"	"
4304	(43 amber)	This lab
4315	"	"
4306	"	"
4312	"	"
4335	"	"
4316	"	"
B22	"	"
4305	"	"
4322	"	"
E192	"	"
4314	"	"
4309	"	"
4302	"	"
R1 - R10	revertants of amNG411	This study
X655	(rIIB opal)	B. Wood
5ts1	(5 ts)	Kao and McClain, 1980
imm2	(imm)	This lab
ocL84	(40 ochre)	"
A104	(40 amber)	Hsaio and Black, 1978b
B49	(40 amber)	"
amN51	(2 amber)	This lab
Phage		
T2	wildtype	B. Wood
T6	wildtype	"

(a) D. Ennis, M. Moran and K. Peterson are at the Univ. of Arizona.

(b) CGSC is the E. coli Genetic Stock Center, Yale University, School of Medicine.

(c) B. Wein is at John Hopkins University, School of Medicine.

(d) BRL is Bethesda Research Laboratories.

(e) J. Wiberg is at the Univ. of Rochester, Medical Center.

(f) B. Wood is at the Univ. of Colorado, Boulder.

(g) The parenthesis under the genotype column in the phage strains indicates the genes bearing the defect and type of defect, if known.

dilution was dispensed into soft top agar along with approximately 0.2 ml of plating indicator, and then spread evenly over the surface of an agar plates by gently tilting the plate in a circular manner. Then the soft agar was allowed to harden on a level surface. Plates were incubated for a minimum of 12 hours at the appropriate temperature and the plaques counted.

Preparation of phage

Phage stocks were prepared by either the plate or bottle lysate method. In the plate lysate procedure 1×10^5 phage and 2×10^9 bacteria were added to 2.5 ml of soft top agar plus 3 ml of HB, and then poured over the surface of a Hershey's plates. After overnight incubation at 30^{o} C several drops of chloroform were added to each plate plus 5 ml of M9 salts solution. Two hours were allowed for the cells to lyse. Then the phage containing fluid was decanted and cellular debris removed by centrifugation at 10,000 x g for 10 minutes. This procedure normally yielded phage titers around 5×10^{10}. If higher titer phage stocks were desired, the phage suspensions were sedimented by centrifugation at 23,000 x g for 2 hours. Then the pellet was resuspended in 1-3 ml of M9 salts solution. This usually resulted in a 10-50 fold increase in titer.

Bottle lysates were prepared by inoculating a 250 ml bottle of HB with 1×10^4 to 10^6 phage and 1×10^8 cfu of host cells. The mixture was incubated overnight at 30 C with aeration. Then several drops of chloroform were added, the mixture stirred vigorously, and let stand for 2 hours. The bacterial debris was removed by a low speed centrifugation as above.

Verification of suppressor host strains

Verification of the presence of a nonsense mutation suppressor in a host strain was accomplished by titering a phage mutant with a known type of nonsense mutation (i.e. amber, opal, ochre). The type of mutant used corresponded to the type of suppressor being tested. A significant increase in the efficiency of plating by nonsense mutants of a particular type on the presumed suppressor strain, as compared to the control strain (known non-suppressor), was taken as evidence for the presence of the corresponding suppressor in the host strain. For example, I used a known opal mutant (X655) in the T4 rIIB gene to verify that E. coli G240R4 and CAJ64 possessed opal suppressors before using them in further experiments.

Standard genetic exclusion assay

The standard exclusion assay measures a primary infecting phage's ability to prevent superinfecting phage from contributing their genetic markers to the progeny. The

quantitative expression of the exclusion phenotype as determined by this assay is referred to as the "immunity value". This is a measure of immunity to superinfection. The immunity value (IV) was determined as follows: The titer of infective centers was measured when a delayed superinfection (D) by a phage T4 amber mutant was carried out after a primary infection by the amber mutant being assayed for its exclusion phenotype. This titer was then divided by the titer of infective centers measured from a simultaneous infection (S) of the same two phage as indicated by the equation:

$$IV = \frac{D \text{ (titer of infective centers after delayed superinfection)}}{S \text{ (titer of infective centers after simultaneous infection)}}$$

Wildtype imm+ phage have an IV of 0.08 at 37°C since the success of delayed infections is much lower than of simultaneous ones. Conversely, the standard imm-2 mutant has an IV of 0.47 at 37°C indicating that in this case delayed infections are more successful.

The procedure for measuring the IV began by growing the host cells to 1-2 X 10^8 /ml at the experimental temperature (ET), and concentrating the host cells by centrifugation at the ET if possible. The hosts were resuspending in prewarmed M9 salts solution (Cloves and Hayes, 1968) to approximately 5 X 10^8 /ml and starved for a

minimum of 30 min. For the delayed superinfection (D), at $t = 0$, 0.1 ml of amber phage #1 (moi $< 10^{-3}$) was added to 1.5 ml of the su- bacteria and the mixture incubated at the ET. [Very low mois (10^{-3} - 10^{-4}) were used to ensure that only single infections occurred, thereby avoiding dosage artefacts.] After 1 min. to allow adsorption, an equal volume of prewarmed double concentration Hershey's broth was added to provide nutrients to permit expression of the exclusion genes. Because complementation or cross-reactivation is required for phage growth, only cells that contain phage #1 and phage #2 will give rise to an infective center. Those cells containing only one of the mutants will not. At t = 8 min amber mutant #2 (having a mutation in a different gene than that of amber phage #1) was added and allowed to adsorb for 4 min before titering on E. coli S/6 (su-). The simultaneous superinfection (S) was carried out in a similar manner except that both amber phages were added at t = 0 min and titered at t = 10 min. A gene 39 double mutant (amber E142, imm-2) was used as an imm- control while a gene 55 amber mutant (NG372, imm+) provided an imm+ control.

Reversion of amNG411

Revertants of amNG411(gene 12) were obtained by plating this mutant at high titer on a non-suppressing host. The plaques that arose resulted from am+ revertant

phage. The reversion rate of amNG411(42) was approximately 2×10^{-6}, so about 1×10^{7} phage were plated on E. coli S/6 (su-) to yield a sufficient number of plaques to test. Ten independent revertant plaques were selected at random, chorded and grown to high titer stocks for further use.

Recombinational mapping of phage mutants

The map position of the T4 mutant amN122(42) is not reported in the literature. I mapped this mutation using the two-factor cross method described by Holmes (1975). The crosses to other previously mapped gene 42 amber mutants were performed in E. coli CR63(su+). To determine the map position of amNG122, it was crossed with amNG385(42) and amE498(42). The map distance from amN122 to amNG385 was 0.1 map units, and to amE498 it was 0.6 map units. This allowed positioning of amN122 at approximately the middle of gene 42. The sum total of the two intervals is 0.7 which agrees exactly with the distance Holmes (1975) found between the two end markers of amNG385 and amE498.

Non-standard exclusion assay

This exclusion assay was employed when the phage being assayed did not carry a conditional lethal mutation, which would be lethal under the assay conditions. I used it to assay the amNG411 revertant phages, since the amber mutation that was formerly in gene 42 was reverted to a non-lethal condition. The assay began by inoculating 1 x

10^9 E. coli S/6 (su-) with the primary infecting phage at an moi of about 0.1. The primary infecting phage is the phage being assayed for its exclusion phenotype. The host cells were previously starved for a minimum of 30 minutes in prewarmed M9 salts solution. After 4 minutes of adsorption (t = 4 minutes) an equal volume of double concentration HB, prewarmed to 37°C, was added to allow expression of the exclusion genes. At t = 8 minutes, amNG205 (gene 42 amber mutant) was used to secondarily infect at an moi of 8 to 10. At t = 12 an aliquot of the culture was sampled for free (unadsorbed) phage and for infective centers to assess the adsorption of the phage to the host cells and their ability to produce productive infections. In order to prevent progeny phage released after a single cycle of infection from colliding with and adsorbing to cells prior to the lysis of the whole population, the superinfected cells were diluted (Ellis and Delbruck, 1939) into 37°C HB within 20 minutes pi. The infected cells were then allowed to incubate for 1 hour. Chloroform was then added to artificially lyse the cells that had not already done so. The progeny phage were plated on E.coli S/6(su-) and on E. coli CR63 (su+). The fraction of the superinfecting phage able to contribute their genetic markers to the progeny was calculated from the plaques formed on each host. The E. coli S/6 strain

titers only the non-amber (am+) phage while the E. coli CR63 strain titers all of the phage in the culture. The plaques formed on E. coli CR63 represent the progeny of both the primary and secondary phage. But the plaques formed on E. coli S/6 represent only the progeny of the primary infecting phage because the secondary phage carries an amber mutation and cannot grow on a su- host like E. coli S/6. The titers on the different hosts were adjusted for the eop of T4D+ (wildtype) on that host for each experiment to allow the results to be directly comparable.

The degree of genetic exclusion was calculated as follows: The eop adjusted titer on E. coli CR63 minus the eop adjusted titer on E. coli S/6 was divided by the eop adjusted titer on E. coli S/6. This number expresses the fraction of superinfecting phage that were able to participate in progeny production. This measures the primarily infecting phage's ability to exclude the secondarily infecting phage. The number is actually the ratio of progeny phage with the secondary infecting phage genotype (2^o) to the number of progeny phage with the primary infecting phage genotype (1^o) or $2^o/1^o$. Imm+ primary infecting phage yield a $2^o/1^o$ ratio of about 1, while imm- primary phages give a ratio about 15 times higher. This is expected since many more secondary

infections can take place in a cell primarily infected with an imm- phage than an imm+ phage.

A variation of this protocol was used when assaying the exclusion phenotype of a primary am, rather am+, infecting phage. In this case the host cell used was E. coli CR63 (su+) which allows growth of the am mutant as well as the secondary infecting am+ phage. The calculations were adjusted to yield the ratio of 2^{o} to 1^{o} infecting phage as before. This variation in protocol was used in assaying the exclusion phenotype of amNG411 to enable direct comparison of this am parent to its am+ revertants. This method provides a convenient measure of the success of a secondary infection without the primary phage having to carry a conditional lethal mutation in an unrelated essential gene as is required in the standard exclusion assay. Despite the apparent simplicity of this assay, it is not the preferred method because it is more difficult to perform and lacks the precision and accuracy of the standard exclusion assay.

Spot tests for complementation and/or marker rescue by a recombinant plasmid

Spot tests generally were performed by the method of Mattson et al. (1977). This method provided a quick and easy way to rapidly screen transformed cells for a recombinant plasmid containing information homologous to

the defective site in a tester mutant. However, this test was not taken as conclusive evidence for such homology. The assay was carried out by the following procedure. The plasmid bearing host was grown to mid-log phase in the presence of the appropriate antibiotic at 37 ^{o}C with aeration. Then 10 ul (microliters) of the culture was spotted on the surface of a plate that had been previously overlaid with soft top agar containing 1 x 10^{9} cells of a restrictive host. While the spots were still wet a 5 ul volume of tester phage (approximately 1 x 10^{6} pfu) was deposited on each spot. The tester phage is usually a phage carrying a conditional lethal mutation in the gene that the transformed cells are being screened for. For example, when testing a recombinant plasmid presumed to have gene 40, I used a phage (ocL84) which has an ochre nonsense mutation in gene 40, and looked for growth (clearing of the indicator lawn under the spot) in restrictive plating conditions. For gene 40 mutants this requires plating at 43 ^{o}C or above on a non-suppressing host, since gene 40 is only essential at high temperatures. The interpretation of the indicator cell lawn clearing patterns is discussed in the results section (Chapter 3).

Quantitative complementation of T4 gene 40 mutants by plasmids

The quantitative complementation method used was a hybrid of those reported by Mattson et al. (1977) and Champness and Snyder (1984). Complementation tests were carried out by measuring phage yields from phage mutant infections of early-log phase plasmid bearing bacterial strains under non-permissive conditions. In theory, if the plasmid has the wildtype gene corresonding to the mutation in the phage and this gene is expressed, then complementation should occur. Early-log phase plasmid carrying strains, grown under antibiotic selection, were infected with phage (moi = 0.1) at 43 °C. This is a restrictive condition for gene 40 mutants, since gp 40 is only essential at high temperature. The phage mutant used was ocL81 (gene 40). The host cells used did not carry an ochre suppressor. At 10 minutes pi an aliquot of the culture was titered for infective centers and unadsorbed phage. The infected cells were lysed after 45 minutes by the addition of a drop of chloroform, shaken vigorously and let stand. After 20 minutes the suspension was diluted and titered for total phage on *E. coli* S/C at the permissive temperature of 30 °C. The average burst size was calculated for each plasmid bearing strain by dividing the number of phage progeny per ml by the number of infected bacteria per

ml. Burst size can be used as a measure of complementation. As controls the mutant was allowed to infect host cells without the plasmid as well as host cell bearing the parental plasmid without the insert.

Quantitative marker rescue of a phage T4 gene 40 mutant

The quantitative marker rescue protocol was adapted from Mattson et al. (1977). The procedure was performed under permissive growth conditions to all ... recombination between the mutant tester phage and an homologous segment of DNA in the plasmid, if it was present. This recombination can lead to rescue of the wildtype marker from the plasmid and hence growth of the recombinant wildtype phage progeny when plated under restrictive conditions. Early-log phase plasmid carrying strains, grown under antibiotic selection, were infected in 1 ml fresh HB with a gene 40 ochre mutant, ocL84, (moi = 1) at 30°C. At 10 minutes pi an aliquot of the culture was titered for unadsorbed phage. At 60 minutes pi one drop of chloroform was added to the culture to lyse the cells. After an additional 20 minutes the culture was titered for total phage on E. coli S/6 at 30°C, the permissive temperature. Wildtype progeny were titered at 43°C, the restrictive temperature. The frequency of wildtype progeny was calculated and used as a measure of homologous recombination by the tester phage with the plasmid insert.

As controls host cells without plasmid and host cell with parental plasmid without an insert were used to establish the background level of reversion of the mutant phage. Any significant increase of marker rescue frequency over the backgound level was taken to indicate the presence of at least part of gene 40 in the plasmid.

Efficiency of plating

The host cells were grown to approximately 5 x10^8 /ml in Hershey's Broth plus antibiotic as required, pelleted by centrifugation and resuspended to the original titer in fresh Hershey's Broth. Approximately 1 x 10^8 cells plus phage were mixed in 3 ml of soft top agar and then plated directly onto enriched HB agar plates. The plates to be incubated at 37 or 43 ^{o}C were prewarmed to 37 ^{o}C prior to plating. The plates were then incubated at the experimental temperature until an adequate indicator lawn was formed. The plaques were tabulated as the mean number per plate $\pm$ the standard error for each temperature category.

Plasmid complementation of the sp genetic exclusion phenotype

In order to screen recombinant plasmids for sp activity, I designed a protocol based on the following logic. Since sp is a phage T1 early gene it should be transcribed by the unmodified E. coli polymerase. When sp

is expressed, it should complement infecting sp- phage. The degree of complementation should then be measurable using the standard genetic exclusion assay. Thus I used the standard genetic exclusion assay performed on the plasmid bearing hosts and determined the recovery (via complementation) of the sp exclusion phenotype of the sp- primarily infecting phage. This was measured as the % recovery of the exclusion ability (IV) in the plasmid-plus-insert bearing cells infected with an sp- primary phage as compared to the exclusion ability (IV) of the same host cells containing the parental plasmid (no insert) infected with sp-. For example, when an sp- phage was assayed in E. coli G240:pBR325(sp-) the IV was 0.142, while an sp+ phage yielded an IV of 0.086, the difference being 0.056. When the same sp- phage was assayed in E. coli G240:pBSK101 (an sp bearing plasmid) the IV was 0.113, a 0.029 decrease in IV from 0.142. This decrease is presumably due to sp gene complementation from the plasmid. The percent change in IV or recovery of the sp exclusion phenotype was calculated as 0.029/0.056 x 100 = 52%. The assays were also done comparing the recovery of the sp exclusion phenotype with a T4 triple mutant, amNG205 imm- sp-. In this case the % recovery of the exclusion ability attained in the plasmid plus-insert bearing cells infected by the triple mutant was compared to the exclusion ability by the same phage in an

infection of the same host bearing the parental plasmid (pBR325). Here, exclusion ability was determined by comparing the amNG205imm-sp- infection to the comparable amNG205imm-sp+ infection.

Subcloning of pBSK101

Once spackle gene activity was detected from pBSK101 the quest began to more precisely define the gene's location. pBSK101 is a 9336 bp recombinant plasmid containing a 3.34 kbp EcoR I fragment of T4 corresponding to the region from 21.155 to 24.495 kbp on the standard T4 restriction map (Fig. 3-7) (Kutter and Ruger, 1985). The fragment was inserted into the EcoR I site of the chloroamphenicol resistance gene of plasmid vector pBR325 (Prentki et al., 1981). pBR325, totalling 5996 bp, also contains the ampicillin resistance (amp^R) and tetracycline resistance (tet^R) genes.

The strategy for locating gene 40 involved the construction of a series of plasmids by systematically deleting portions of the T4 insert carried by pBSK101. Each plasmid was then assayed for sp activity. See Fig. 2-1 for the genealogy of the plasmids and Fig. 3-7 for an expanded version of the T4 restriction map.

1. Determination of the insert orientation. Since the T4 fragment in pBSK101 carried EcoRI homologous ends and was inserted into a pBR325 plasmid with the same ends,

the insert could be oriented in either direction. To determine the orientation of the insert a Pst I digest was performed on approximately 500 ng of pBSK101 DNA. The resulting fragments were sized by gel electrophoresis (Maniatus et al., 1982) in 1% agarose (FMC) with a 0.5x Tris Borate buffer system using DNA molecular weight markers from Bethesda Research Laboratories (BRL). The fragment sizes (3164 and 6172 bp) indicated the orientation of the insert to be as depicted in Fig. 2-1.

Between major manipulations of the DNA such as endonuclease restriction, ligation, etc. the DNA containing solution was extracted twice with phenol (pH 7.6, equilibrated with 0.5M Tris), once with a 1:1 mixture of phenol and chloroform (1/24 isoamyl alcohol) then thrice with an equal volume of water saturated ether. After ether extraction the solution (uncapped) was placed in a 65 °C water bath for 10 minutes to allow the remaining ether to evaporate. The DNA was concentrated by the method suggested by BRL. This involves the addition of 1/10 volume of 2.5M (pH 5.2) sodium acetate (or 1/5 volume of 10.5 M ammonium acetate) plus a 2 fold volume of reagent grade ethanol. The solution was then placed in wet ice for 15 - 20 minutes and centrifuged for 30 minutes in an Eppendorf centrifuge (or 12000 x g) at 0 °C. The pellet was washed 2 or 3 times with 70% ethanol. Most of the wash solution was removed

Fig. 2-1: The above diagram schematically represents the subcloning of pBSK101. The underlined numbers around the diagram of pBSK101 are the bp locations of the restriction sites of the 5996 bp parental plasmid, pBR325 (Prentki et al., 1981). The numbers in ( ) represent the size of the T4 DNA insert. The number under the plasmid designation is the total plasmid size in bp. The 5-digit numbers correspond to the restriction site locations in kbp on the T4 restriction map (Fig. 3-7) (Kutter and Ruger, 1985).

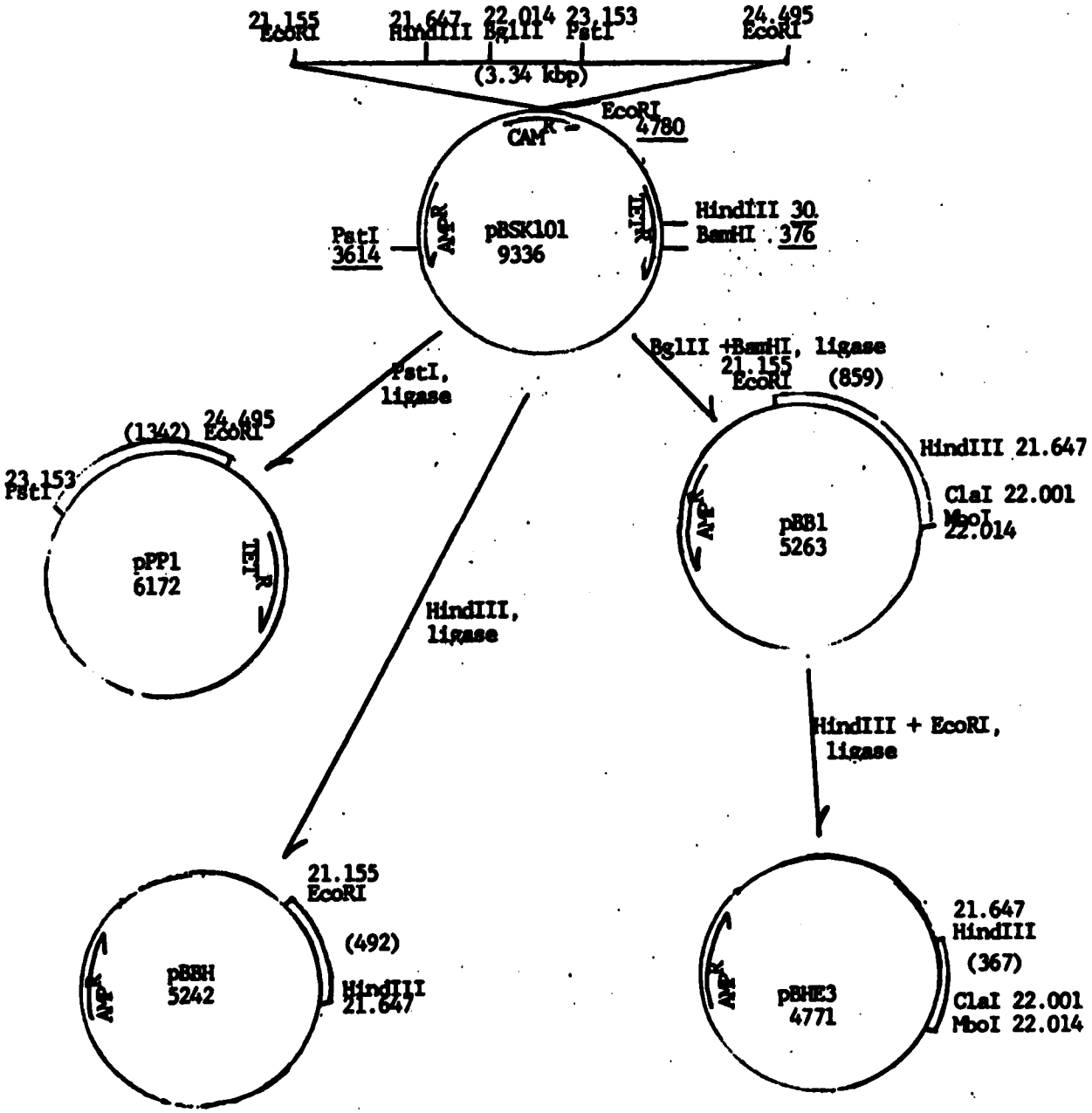


Fig. 2-1: Subcloning of pBSK101

from the pellet by pipetting, with the remainder removed by a brief exposure in a vacuum desiccator. All enzymatic reactions were performed according to the supplier's recommended conditions.

2. Construction of pPP1. Plasmid pPP1 was constructed by deletion of a 2.14 kbp region from pBSK101. The deletion corresponded to an area from 21.155 to 23.153 on the T4 restriction map leaving a 1342 bp fragment in the new pPP1 plasmid between 23.153 to 24.495 on the T4 map. A portion of the vector was also removed in the process. This was accomplished by a complete digestion of pBSK101 with Pst I resulting in two fragments of sizes 6172 bp and 3164 bp. After extraction the fragment containing mixture was treated with T4 ligase (BRL) at room temperature.

Host cells, E. coli G240, were made competent by the calcium chloride procedure (Maniatus et al., 1982) and transformed with the ligation mixture. Theoretically, the ligation mixture contained an assortment of the following circularized DNA species: religated pBSK101; the 3161 bp fragment that does not contain an origin of replication (ori); the desired 6172 bp ori and tet^R containing fragment; and the 6172 bp fragment with the 3161 bp fragment in the reverse orientation as compared to pBSK101, plus a few others of lesser significance. The competent cells were transformed (Maniatus et al., 1982) and plated

on tetracycline (tet) plates. Only cells transformed with DNA containing an ori and the tet^R gene could grow. The resulting colonies were then picked onto ampicillin (amp) plates and tet plates. Those colonies that grew on both were discarded as they were probably pBSK101. Several colonies showing only tet resistance were grown in LB plus tet [15 micro(u)g/ml] (Maniatus et al., 1982) to stationary phase at 37 °C. Using the alkaline lysis mini plasmid preparation procedure (Maniatus et al., 1982) the plasmids contained in the tet resistant colonies were extracted and their size determined using an agarose gel electrophoresis procedure in which the DNA was stained with ethidium bromide and visualized with long-wave ultraviolet light. This distinquished the desired 6172 bp plasmids from those 9336 bp plasmids having the 3164 bp Pst I fragment inserted in the opposite direction than pBSK101. The 6172 bp plasmids were further restricted with Mlu I, doubly digested with combinations of Mlu I/Nde I and Pst I/EcoR I to confirm the clone as having the correct insert. Refer to Fig. 3-7 for an expanded T1 restriction map of the region that was used to plan restriction strategies to confirm the correctness of the plasmid inserts.

3. Construction of pBB1. Plasmid pBB1 was subcloned from pBSK101 and contains a fragment of T1 DNA corresponding to a region from 21.155 to 22.011 kbp on the

T4 map. It was constructed by doubly digesting pBSK101 with Bgl II and BamH I and religating after phenol extraction. The desired plasmid has an ori and amp resistance with a total size of 5263 bp. E. coli G240 cells were transformed as above and selected on amp plates. Tet resistant colonies were discarded and mini plasmid preparations were done to isolate the correct recombinant plasmids from the remainder. Plasmids of the correct size were doubly digested with EcoR I and Cla I. Those yielding a fragment of 846 bp were selected as the correct construct and one was designated pBB1.

4. Construction of pBHE3. Plasmid pBHE3 is a product of a double digest of pBB1 with Hind III and EcoR I followed by a 2 bp partial homology ligation of the ends to recircularize the plasmid. Competent E. coli G240 were transformed with the ligation mixture and plated on amp containing plates. Mini plasmid preparations from the transformants were used to determine plasmid size as a means of confirming the identity of the insert. They were also tested for the expected restriction sites by double digestion with Cla I and Pst I to confirm the identification. A transformant, designated pBHE3, yielded the predicted fragment sizes of 1520 and 3251. Its plasmid contains a 367 bp insert of T4 DNA corresponding to a region from 21.647 to 22.014 on the T4 restiction map.

5. Construction of pBBH. Plasmid pBBH was constructed by the deletion of 2.85 kbp of the 3.31 kbp T4 insert in pBSK101. pBSK101 DNA was digested to completion with Hind III, the solution extracted and treated with ligase to circularize the plasmid using the Hind III homologous ends. Competent E. coli G210 cells were transformed and selected for amp resistance. Transformants were grown in HB to stationary phase and then their plasmids were extracted for determination of size. Those transformants with the correct size plasmid were further restricted by double digestion of EcoR I and Hind III. The predicted 492 bp fragment was visualized in the electrophoretic pattern of the digested pBBH. The 492 bp fragment maps to a region from 21.155 to 21.647 on the T4 restriction map.

6. Construction of pJO11. Plasmid pJO11 is a recombinant plasmid of pUC18 with a 367 bp insert of T4 DNA from the region 22.014 to 21.647 kbp on the T4 restriction map. I made this recombinant plasmid by first preparing the plasmid expression vector. This involved digesting it with both Hind III and BamH I. After digestion the reaction mixture was extracted and prepared for the ligation reaction.

Next the 367 bp T4 insert was removed from pBSK101 by double digestion with Hind III and Bgl II. The Hind III

end was intended to match its complement in the vector while the Bgl II end matched the BamH I end in the vector. This arrangement allowed for the forced cloning of the fragment in the correct transcriptional orientation for gene expression. The 367 bp fragment was isolated by gel electrophoresis in a TBE buffer system in 1% low-gelling temperature agarose (SeaPlaque, produced by Marine Colliods, FMC) containing 10 mg/ml ethidium bromide. For determining the size of the low molecular weight DNA fragments a 123 bp ladder sizing standard marker was used (Sigma). The 367 bp fragment was visualized with long-wave ultraviolet light and excised with a clean razor blade. The gel slice was melted at 65 - 70 °C for 5 - 10 minutes and then transferred to a 42 °C water bath until used.

In a separate tube maintained at 42 °C, I mixed the T4 DNA insert in an estimated 3:1 molar ratio with the prepared vector to a final volume of 10 ul. To this mixture I added a 2x concentrated ligation buffer containing 10 units of T4 ligase, mixed quickly and incubated the mixture at room temperature overnight (method modified from Struhl, 1985). Since the ligation is done in the presence of agarose, the mixture hardens at room temperature.

The next morning I treated E. coli DH5 (Φ80dlac Z del M15, r-) cells to promote competence using a method

modified from Hanahan (1985). I found this rubidium chloride method to increase the efficiency of transformatiom 1000 fold over the calcium chloride method used previously (data not shown).

To transform the competent cells, I remelted the ligation mixture and diluted it by a factor of 10 with ice-cold TCM (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$). Using 10 ul, 20 ul, 50 ul of the solution I transformed 3 tubes of competent E. coli DH5 using the transformation procedure suggested by Hanahan (1985). From each tube, one third of the volume was added to each of 3 amp plates containing IPTG (isopropylthiogalactoside) and X-gal(5-bromo-4-chloro-3-indolyl-Beta-D-galactoside). After overnight incubation at 37 ^{o}C several white colonies were selected for further screening.

Plasmid pUC18 is an expression vector containing the amp^{R} gene and a portion of the lac Z gene (Beta-galactosidase) with a multiple cloning site (MCS) between the gene and its controlling elements. When the MCS is intact and inducing conditions are used (addition of lactose or IPTG) the lac Z gene is turned on and produces Beta-galactosidase (B-gal). B-gal has the ability to react with X-gal (chromogenic substrate of B-gal), which bestows a blue color to the colony of cells containing pUC18. However, when a piece of foreign DNA is inserted in the

MCS, this usually results in loss of B-gal activity resulting in white colonies in the presence of X-gal in lac Z- host cells. Therefore, white transformants on X-gal/IPTG/amp plates usually means a plasmid with insert due to the insertational inactivation of the lac Z gene of the plasmid.

As previously mentioned several white colonies were selected and grown to stationary phase in HB. Their plasmids were extracted and the size of the plasmids was determined as before. Those with the correct size were futher analyzed using restriction endonucleases. The candidate plasmids were first digested with Hind III expecting linearization. They were then doubly digested with the followint three pairs of enzymes: Hind III/EcoR I, Hind III/Cla I and Nde I/EcoR I. The critical fragment sizes expected were of 390, 354 and 605 bp respectively. Since plasmid pJO11 gave these expected results, I concluded it contained a 367 bp fragment mapping to the 21.647 to 22.014 kbp region of T4 in the proper orientation in the vector.

Preparation of cell lysates for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

E. coli DH5:pJO11 and the control cells without the T4 insert were grown to approximately 2 x 10 /ml with antibiotic selection under inducing conditions (in the

presence of IPTG or lactose). Then 1 ml of the culture was pelleted in an Eppendorf centrifuge, and washed twice with an equal volume of M9 salts solution. The pellet was next resuspended in 200ul of SDS-PAGE loading buffer which contained 0.05 M Tris-HCl, pH 6.8, 1% SDS, 1% beta-mercaptoethanol, 10% glycerol and 0.05% bromphenol blue as a tracking dye. The suspension was boiled for 5 minutes, cooled and then 20 ul of each separate sample was loaded on the electrophoresis apparatus per lane.

SDS-PAGE Analysis

The SDS-PAGE was carried out using a modification of the method of Laemmli (1970). The Biorad SDS-PAGE Protean apparatus with a discontinuous buffer system was used with a slab gel composed of a 4.5% stacking gel and a 15% separating gel (200mm x 200mm x 0.5mm). Electrophoresis was conducted at 300 constant volts until the dye reached the separation gel, and then the voltage was increased to 500 volts. The 20 ul samples (E. coli DH5, E. coli DH5:pUC18, and E. coli DH5:pJO11-induced) were loaded, one per lane, with standard molecular weight markers (BRL) on either side of the three sample lanes. The gels usually ran for 4 to 4.5 hours. After electrophoresis the proteins were fixed and stained with a solution of 0.2 % Coomassie brilliant blue in 50 % methanol and 10 % acetic acid for approximately one hour. The gels

were then destained in the above solution without the stain until the background stain disappeared. A 15 % gel system was selected because of the possible small molecular weight(s) of the protein(s) produced by the recombinant plasmid.

CHAPTER 3

RESULTS

The imm Gene

Characterization of the imm-2 mutant

The imm gene mutant isolated by Vallee and Cornett (1973) was designated as imm-2 and it proved to be allelic with Childs' (1973) mutant, designated as imm 1. In further work imm-2 was used as our standard imm- mutant. The imm-2 mutation is not suppressible in amber suppressor containing hosts, so imm-2 is not an amber mutation (Vallee and Cornett, 1973). [The three nonsense or termination codon mutation designations are: (i) amber having a UAG sequence on the messenger RNA (mRNA). (ii) Opal - UGA. (iii) ochre or umber - UAA (Birge, 1981).]

To facilitate future investigation of the imm gene's involvement in genetic exclusion, I attempted to further characterize the imm-2 mutation. Using the standard genetic exclusion assay designed to quantitate the degree of exclusion of the primarily infecting phage, the imm-2 mutant was assayed in various E. coli hosts and at a range of temperatures. Briefly, the assay yields a value, henceforth referred to as the immunity value (IV), that reflects the amount of successful infection by the

secondarily infecting phage. Therefore low IV, indicates a low rate of successful superinfection, i.e. a high level of exclusion. A high IV, indicates a successful superinfection, i.e. a defective exclusion phenotype. For example a phage that is wildtype for exclusion characteristically yields an IV of 0.08 at 37°C in E. coli S/6, whereas imm-2 yields an IV of 0.47 due to the increased success of superinfection. The IVs can vary slightly depending upon the host.

To ascertain whether or not the imm-2 mutant had an opal or ochre defect, I assayed its ability to genetically exclude superinfecting phage in various E. coli nonsense suppressor strains. If imm-2 is suppressed by the suppressor carrying strain the resultant IV would be expected to move from that of the imm- phage to that of the imm+ phage. As can be deduced from the results presented in Table 3-1, the imm-2 mutation present in the strain amE112(gene 39), imm- is not an opal (UGA) suppressed by the insertion of tryptophan, nor is it an ochre/amber mutant (UAA/UAG) suppressed by the insertion of lysine or tyrosine. In no case did the imm mutant exhibit a wildtype phenotype. Since the mutation in the imm-2 mutation was not suppressed it probably is not a nonsense mutation, at least one corrected by the insertion of the above amino

acids. In my succeeding experiments with imm-2 I took this conclusion into account.

The last column in Table 3-1 shows data for amNG111(gene 42) which has a defective exclusion phenotype and is used here as an imm- control. I will return to these results after reviewing the data in Table 3-2.

The exclusion phenotype of gene 42 and 43 amber mutants

As mentioned in Chapter 1, there is an inconsistency between the phage T4 restriction map and the reported imm gp molecular weights. When the reported sizes for the imm gene product (gp) are compared (Chapter 1) with the coding region available between genes 42 and 43, one finds that there are insuffient base pairs to code for the entire imm gp. A conservative explanation is that the imm gene overlaps an adjacent gene. Two lines of evidence suggest that this is the case. First, Childs (1973) using partial T4 genomes as the basis for mapping only obtained the imm+ phenotype when a gene 42 marker was also present in the phage. Second, Lu (1982) reported screening 5000 clones from a phage T4 genomic library and finding at least a portion of gene 42 on every one containing the imm gene. Furthermore, a strong early promoter has been mapped to the reputed imm gene region (26.3 kbp) providing a likely candidate for the imm gene's regulatory element. If an overlap exists, a nonsense mutation of a neighboring gene

Table 3-1: Genetic Exclusion by Three Phage Strains in Various E. coli Nonsense Codon Suppressor Hosts.

E. coli Strain	Phage T1 Strain amNG372 (gene 55, imm+)	amE112 (gene 19), imm-	amNG111 (gene 12)*
U11R1d (Su 5, lysine- UAA-UAG)	.11±.01	.39±.02	.31±.01
H12R7a (Su C, tyrosine- UAA-UAG)	.12±.03	.57±.01	.39±.01
CAJ64 (Su 9, tryptophan- UGA)	.20±.01	.29±.02	.58±.06
G210R4 (Su 9, tryptophan- UGA)	.12±.02	.52±.04	.13±.05

The above numbers represent the average Immunity Value obtained by the standard genetic exclusion assay at 37oC for each phage strain plus or minus the standard error. *Phage amNG111(gene 12) exhibits a defective exclusion phenotype.

located in the shared region may also appear as a mutation in the imm gene. Therefore, I assayed amber (UAG) mutants defective in genes 42 and 43 (the neighbors bordering imm) for their effect on the imm phenotype. If the genes overlap in the same reading frame, a nonsense mutation in one gene will also be a nonsense mutation in the other. On the other hand if the overlaps are out-of-phase then a nonsense mutation in one gene (either gene 42 or 43) may introduce a missense mutation in the other (imm). In this case, the different amber mutations in the overlap region would be expected to have different effects on the imm phenotype. Some of the resultant missense mutations in the imm gene might be silent, and some might be temperature or cold sensitive. Others might cause a mild perturbation in the imm gp structure with a small functional loss, while others may result in a totally defective protein and a corresponding imm- phenotype.

Because of the expected variation, I screened 17 amber mutations in gene 42 and 13 in gene 43, which mapped along nearly the entire length of each gene (Fig. 3-1), at various temperatures. As a positive control an imm+ phage with an amber mutation in an unrelated gene 55 was used, and as a negative control an imm- mutant with a second amber mutation in gene 39 was used. The second amber mutation is required for the experimental protocol but has

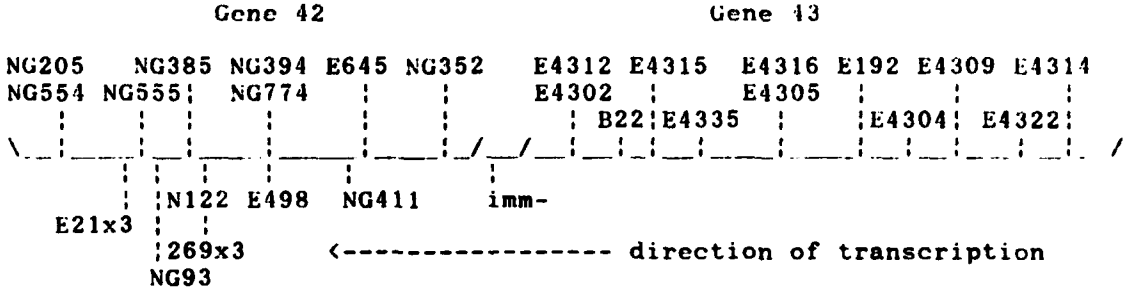
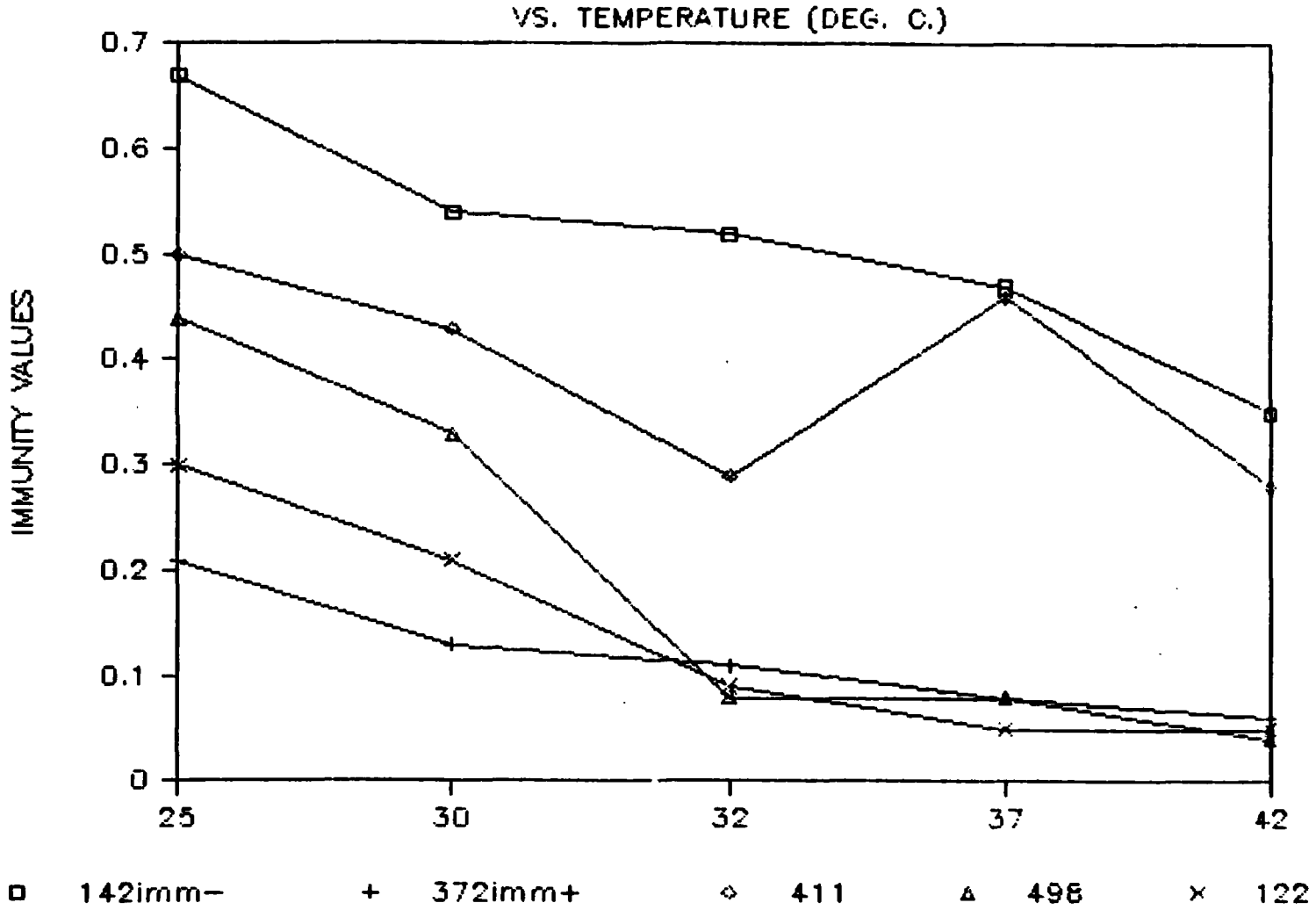


Fig. 3-1: A genetic map of the amber mutation sites in genes 42 and 43 (plus the imm gene). All mutants that were assayed for their immunity phenotype are indicated according to the site of their defect. The map positions of the gene 43 mutants were taken from Reha-Krantz and Bessman (1981) and Allen, Albrechet, and Drake (1970). The map positions of most of the gene 42 mutant sites were taken from Holmes (1975) with the exception of amN122(gene 42), which I mapped using the two-factor cross method described by Holmes. The positions of E21x3 and 269x3 that were kindly provided by J. S. Wiberg (personal communication, 1986). The mutant designations appearing above the horizontal line displayed an imm+ phenotype, while those below exhibited some degree of defective immunity. Two other gene 42 mutants, CT36, E117 which were not mapped also showed an imm+ phenotype, while phage C87, also unmapped, showed a slightly defective imm phenotype.

TABLE 3-2. Immunity of gene 42 and gene 43 amber mutants

Gene(s)	Amber Mutant	Immunity values obtained at the temperatures indicated				
		25°C	30°C	32°C	37°C	42°C
39, *imm*-2	E142	.67±.05	.54±.03	.52±.03	.47±.03	.35±.03
55, *imm*$^{+}$	NG372	.21±.01	.13±.01	.11±.01	.08±.01	.06±.01
42	NG411	.50±.03	.43±.02	.29±.01	.46±.08	.28±.05
	E498	.44±.02	.33±.05	.08±.00	.08±.00	.04±.01
	N122	.30±.02	.21±.01	.09±.01	.05±.01	.05±.01
	NG93		.21±.03		.15±.01	
	E21x3		.21±.02		.17±.03	
	C87		.19±.04		.10±.01	
	269		.18±.03		.07±.01	
	NG205		.14±.03		.04±.01	.08±.02
	E117		.13±.02		.05±.01	.06±.02
	E774		.12±.01		.06±.01	.07±.02
	NG555	.22±.01	.11±.01		.12±.02	.05±.01
	NG554		.11±.00		.06±.01	.07±.01
	CT36	.23±.02	.11±.02		.18±.03	.04±.00
	E645	.24±.00	.10±.03		.11±.02	.04±.00
	NG394	.22±.02	.10±.01		.09±.02	.06±.00
	NG352		.07±.00		.05±.00	.05±.01
43	4304		.15±.01		.09±.00	.10±.01
	4315		.15±.03		.04±.01	.05±.00
	4306		.13±.02		.04±.00	.04±.01
	4312		.12±.07		.09±.01	.05±.00
	4335		.11±.01		.05±.00	.06±.01
	4316		.10±.02		.05±.01	.04±.00
	B22		.10±.01		.05±.01	.04±.01
	4305		.10±.01		.10±.02	.07±.01
	4322		.09±.01		.06±.02	.05±.00
	E192		.09±.03		.05±.00	.05±.00
	4314		.09±.01		.03±.00	.05±.00
	4309		.07±.01		.04±.01	.05±.01
	4302		.01±.01		.04±.01	.04±.00

Fig. 3–2: IVs OF GENE 42 & 43 MUTANTS



been shown to have no affect on the immunity phenotype (Vallee and Cornett, 1973). The protocol for the standard genetic exclusion assay is described in Chapter 2. The results in Table 3-2 show that seven amber mutants, all in gene 42, are significantly defective in imm function. One (amNG411) was defective at all five temperatures, while amE498 and amN122 were measurably defective at 25°C and 30°C, but not at 32°C and above. Two others (amNG93 and amE21x3 were defective to an intermediate degree with no observed temperature sensitivity. Finally, amQ87 and am269x3 are significantly defective in imm function only at 30°C. Fig. 3-2 provides a graphic representation of the IVs of three exclusion defective gene 42 mutants compared to the imm+ and imm-2 controls. The figure shows the consistent imm- phenotype of amNG411(42) at all temperatures and the cold sensitive phenotype of E198(42) and amN122(42). Amber mutations which do not exhibit a defective imm phenotype are interspersed among those that do (Fig. 3-1). Therefore genes 42 and imm probably do not overlap in the same reading frame, but rather overlap in an out-of-phase orientation. Assuming the recombinational map of gene 42 represents the actual linear order and approximate spacing of the sites of the mutations, the imm gene shares approximately 50% of gene 42's base pairs.

Additionally, by comparing the row of IVs in Table 3-2 for phage E142(39),imm-2 to IVs for the imm+ contol (NG372), one can see that imm-2 is not a temperature sensitive or cold sensitive mutant.

The exclusion phenotype of amNG411 revertants

One possible explanation for the strongly defective exclusion phenotype of amNG411(gene 42) is that it is a double mutant; that is, it may have an amber mutation in gene 42 and a second mutation in a non overlapping imm gene. To investigate this possibility I selected 10 independent spontaneous revertants of amNG411 based on growth of the phage on a host lacking an amber suppressor. I then assayed these am+ revertants for their exclusion phenotype. As controls, the amNG411 parent as well as imm+ and imm-2 phage were tested for their exclusion phenotype. The technique I employed was developed by S Abedon (personal communication, 1986, see chapter 2). Its advantage for measuring the exclusion phenotype of the revertants is that it does not require an unrelated conditional lethal mutation in the phages being assayed. This non-standard exclusion assay measures the exclusion phenotype of the primarily infecting phage as the ratio of the secondary infecting phage's growth in the presence of the primary phage to its growth in the absence of the primary phage. Under the assay conditions used, this ratio is found when the primary phage is imm+ is at least

Table 3-3: Genetic Exclusion by amNG411 (gene42) Revertants at 37oC

Phage strain	$2^{o}/1^{o}$ +SE
imm+ (T4D+)	0.65±0.16
R1	1.36±0.04
R2	1.07±0.15
R3	0.90±0.22
R4	1.60±0.36
R5	1.08±0.25
R6	0.99±0.13
R7	0.88±0.06
R8	1.07±0.10
R9	1.26±0.38
R10	1.07±0.35
imm-2	3.65±1.35
amNG411 (parent)	3.96±0.21

The above values are measures the exclusion phenotype of the designated phage using the non-standard genetic exclusion assay. The assays of the amNG411 revertants (designated R 1 - 10) were performed in E. coli S/6(su-) at 37oC. The amNG111 parent was assayed in E. coli CR63(su+) because the gene 12 amber mutation would intefere with the progeny production in a Su- host. The imm+ and imm-2 controls were tested on both E. coli S/6 and CR63. The values of amNG111 determined on E. coli CR63 were normalized to the E. coli S/6 scale for the purposes of direct comparison.

(Table 3-3), while the value for primary imm-2 phage was 3.65. This greater ratio reflects an increase in the proportion of the genotype of the secondary phage in the progeny when the imm gene is defective. (Note that amNG111 is also exclusion defective in this assay yielding a ratio of 3.96.) The $2^{o}/1^{o}$ ratios of the amNG111 revertants are intermediate, but more closely resemble the $2^{o}/1^{o}$ ratio of imm+ than of imm-2 or the amNG111 parent (Table 3-3 and Fig. 3-3). These results demonstrate conclusively that when the amber mutation in amNG111 is reverted for the gene 12 conditional lethal phenotype, the imm- character also reverts to a more nearly imm+ phenotype. These are exactly the results predicted if amNG111 does not contain a second mutation, and the amber mutation in gene 12 is the same mutation that exhibits a defective exclusion phenotype. If amNG111 was actually a double mutant, the chance of all 10 revertants having spontaneously reverted at both sites is approximately $1/10^{22}$. These results not only indicate that amNG111 does not have a second site mutation in the imm gene (or any other exclusion related gene), but also provide further evidence that genes 12 and imm overlap.

The effect of opal suppression on exclusion

Preliminary DNA sequence information, done with me by H. Krisch (personal communication, 1985) shows a long open reading frame (ORF) extending from a point in the

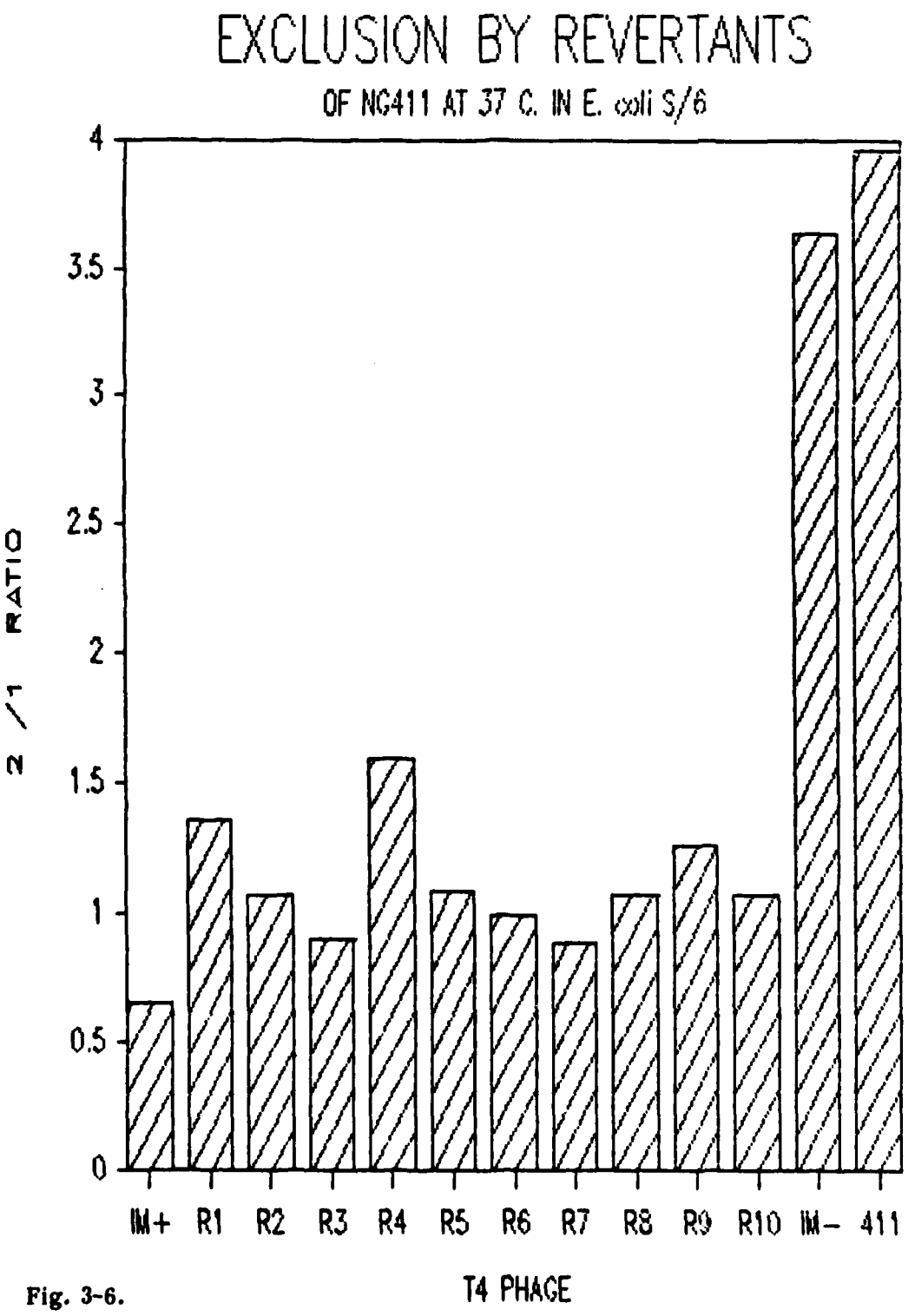

EXCLUSION BY REVERTANTS
OF NG411 AT 37 C. IN E. coli S/6
2 / 1 RATIO
4
3.5
3
2.5
2
1.5
1
0.5
0
IM+
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
IM-
411
T4 PHAGE

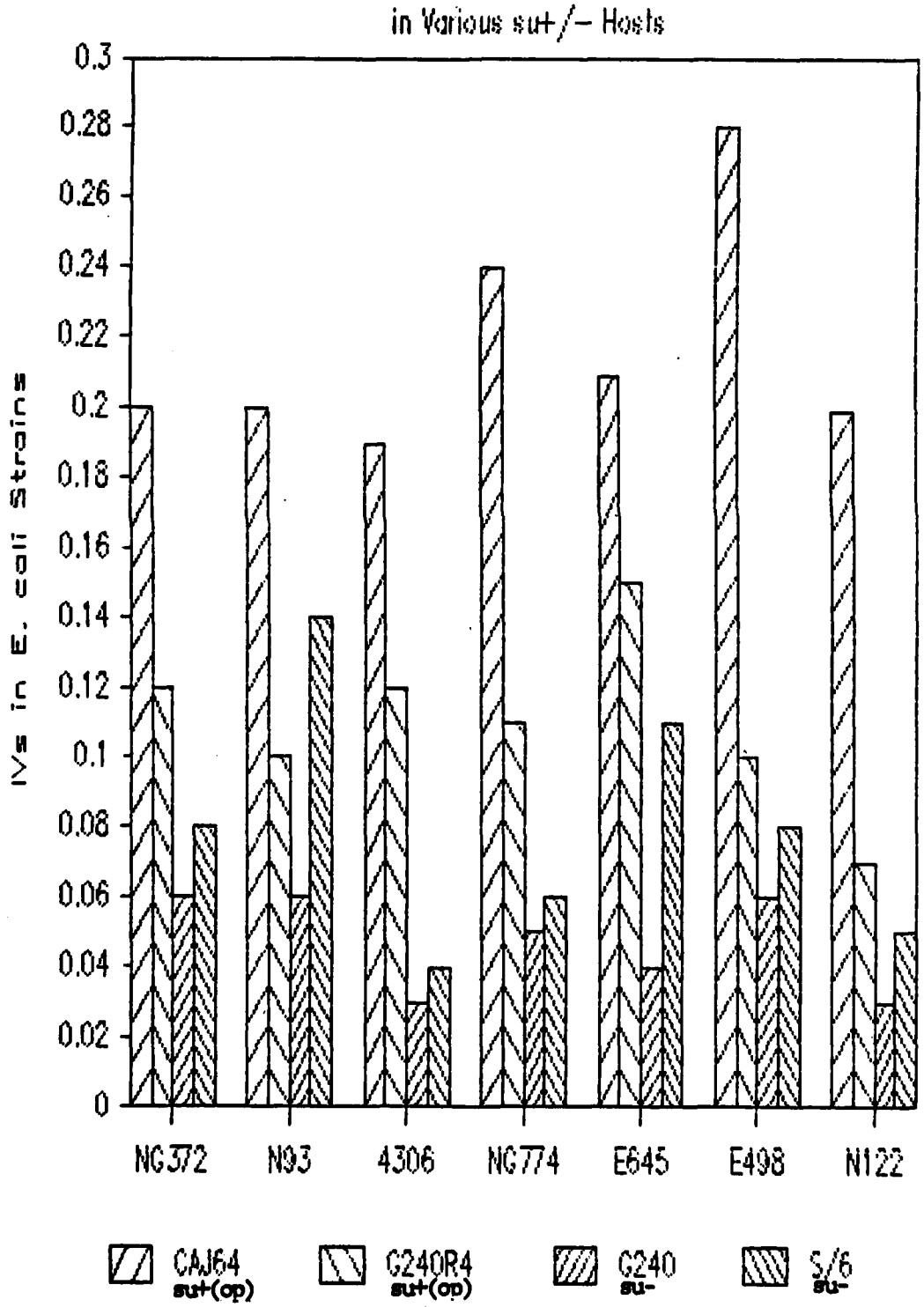
Fig. 3-6.

putative imm region into structural gene 42 in an out-of-phase orientation. If it were not for a single UGA (opal) termination codon 256 base pairs into the imm gene ORF and prior to the gene 42 promoter region, I would consider this conclusive evidence of the hypothesized overlap. At first glance the presence of the termination codon, if his sequence data is correct, would negate the proposed overlap, but there is considerable precedent in the literature for read-through of termination codons (Garen, 1968; Philipson et al., 1978; Geller and Rich, 1980; Birge, 1981), specifically opals (Garen, 1968; Weiner and Weber, 1971; Yates et al., 1977; Nasmyth and Tatchell, 1980), especially in phage (Weiner and Weber, 1973; Yates et al., 1977). As a matter of fact an opal termination codon read-through hypothesis would nicely account for the here-to-fore inexplicable observations by two independent laboratories (O'Farrell and Gold, 1973; Yutsudo, 1979) that imm is responsible for two proteins. This could be accounted for if there were a less than 100% efficient read-through of the opal codon, as seen in the phage lambda [illegible] gene (Yates et al., 1977). Although the reported molecular weight [illegible] between the two [illegible] of the large imm protein to the [illegible] (O'Farrell and [illegible]).

To address the subject of an opal (UGA) nonsense codon in the sequence of imm prior to gene 42, I designed an experiment based on the following logic. If the opal termination codon is read-through to produce a functional imm gp then growing imm+ phage T4 in an E. coli opal suppressing host may result in a measurable change in the imm phenotype. The two opal suppressing strains used insert the amino acid tryptophan (trp) at the site of the opal termination codon. In one case the su- parent was available as a control, in the other I could not obtain the isogenic su- parent. There are three possible outcomes of preferentially inserting trp in place of the opal codon: (i) trp is the amino acid inserted by the "normal" read-through mechanism so that no phenotypic change will result, (ii) trp is not the usual amino acid at this position, so that insertion of trp improves the function of the imm gp, resulting in a qualitative increase in imm function which may or may not be measurable; (iii) trp is not the usual amino acid at this position, so that its insertion causes some degree of impaired function which can be measured.

Using E. coli C21084 (Su 9, trp), an opal suppressor strain, I found a small but statistically significant increase in IV (decrease in immunity) for each of 7 mutants previously determined to be imm+, compared to the immunity value obtained by the same phage in the suppressor minus

Fig. 3-4: Immunity Values of T4 Mutants



parent strain E. coli (G240) (Fig. 3-4). In E. coli CAJ64, the other UGA suppressor strain, the immunity values were 2-3 fold greater than the values obtained in the su- hosts of E. coli G240 or E. coli S/6 implying an even greater loss of immunity (Fig. 3-1). E. coli S/6 was the su- minus host used in the gene 42 and 43 mutant screening experiments (Table 3-4). In general these results show a decrease in exclusion of imm+ phage in the presence of an opal suppressor. They can be explained by reasoning that the trp inserting opal suppressor competes with the normal read-through mechanism to yield at least some imm gp which is defective due to the trp substitution. These results indicate that an opal termination codon is involved in the full phenotypic expression of the imm gene, and taken in light of the sequence data they further support the hypothesis that the imm gene overlaps with gene 42.

Thus far I have obtained strong genetic evidence for the existence of an out-of-phase overlap between the imm gene and gene 42 with probable involvement of an opal termination codon.

Cloning of the imm gene

In order to provide molecular proof of the overlap between the imm gene and gene 42, I attempted to clone the imm gene on an inducible-expression vector plasmid to

demonstrate its biological activity, and then to define the overlap by in vitro directed mutagenesis. After multiple attempts using a variety of hosts and plasmid vector systems I was unable to clone the imm gene.

The effect of exonuclease V in genetic exclusion

There has been much speculation concerning the mechanism of imm gp action in the exclusion process. Some inferential evidence has been presented (Vallee and Cornett, 1973; Vallee and de Lapeyriere, 1975; Yutsudo and Okamato, 1973) but little solid molecular or genetic findings have been obtained. The general consensus is that gp imm acts at the level of the membrane, either altering a phage adsorption site or interfering with DNA ejection or uptake. However, the possibility that the imm gp may be involved in a cytoplasm-based exclusion response has not been considered in the literature.

Exonuclease V (exo V) is a cytoplasmic enzyme produced by the E. coli rec BCD locus. It has several functions but its ability to degrade DNA from the 5' end is regarded as its most biologically significant activity (reviewed in Telander-Muskavitch and Linn, 1981; Amundsen et al., 1986). Amber mutants defective in gene 2 and 61 when grown in a restrictive host form progeny which can adsorb normally to the host and kill it, but could not produce a productive infection (Granboulan, Sechaud and Lellenberger, 1968;

Silverstein and Goldberg, 1976a). The host exoV was shown to degrade phage T4 5-hydroxymethylcytosine containing DNA (HMC-DNA) in these infections. However, gene 2 and 64 mutants do form plaques on exonuclease V defective hosts. Genes 2 and 64 are now thought to be one gene (rather than two) which presumably acts to protect the phage DNA from exonucleolytic degradation (Goldberg, 1983).

I reasoned that one strategy by which primary phage might exclude superinfecting phage DNA would be to render it susceptible to host exonucleases. It would seem the simplest and most economical way to accomplish this would be to inactivate the protective mechanism of the superinfecting DNA. To pursue this, I first addressed the question of whether exonuclease V is involved in the genetic exclusion process at all.

I performed the standard exclusion assay on several phage strains to investigate the above question. Two were imm+ and sp+ (one carried the an amber muation in gene 53; the other in gene 42). I also used an imm-2 mutant, a sp- mutant and an imm-2, sp- double mutant. (All of the imm- and/or sp- mutants had the same amber mutation in gene 42 as the imm+ strain, thus providing a consistent genetic background for comparison purposes). The assays were performed in strains of E. coli that were either wildtype or defective for exonuclease V. E. coli 591 is wildtype for

exo V and the parent of DE828. DE828 carries a rec D mutation rendering it completely defective in exo V activity (Amundsen et al., 1986). E. coli JC5519 is another exo V defective strain having mutations in the rec BC locus. As can be seen from Table 3-1 when the host is exoV- there is a substantial increase in IVs in the standard exclusion assays where the primary infecting phage was imm+ compared to the IVs obtained in the exo V+ host strain. The average increases are 2.6 fold for the two imm+ sp+ strains, and 1.4 for the imm+ sp- strain. The most reliable comparison is between the values obtained in E. coli 594 and the isogenic DE828 strain. Collectively these results show that when the phage are imm+ there is decreased exclusion (i.e. increased IV) in the exonuclease V deficient strains. This demonstrates that exonuclease V is involved in the exclusion process. And it also suggests that gp imm alters the secondarily infecting phage's incoming DNA to expose it to exonuclease V degradation. On the other hand sp gp seems to have no involvement in exo V mediated exclusion.

The effect on exclusion of an additional endo I mutation.

Also in Table 3-1, the effect on exclusion of exonuclease V and endonuclease I (endo I) host mutations on exclusion can be evaluated. E. coli strains MP3-9 and CES201 contain both a recBC mutation and an sbcB mutation

Table 3-4: Genetic Exclusion by Phage T4 imm+/-, sp+/- Strains in Various E. coli Hosts (Endonuclease I +/- and Exonuclease V +/-).

T4 gene	mutant			E. coli hosts				
				594	DE828 (594,recD)	JC5519 (recB,C)	KP360 (recB,C; sbcB)	CES201 (recB,C; sbcB)
				exoV+ endoI+	exoV- endoI+	exoV- endoI+	exoV- endoI-	exoV- endoI-
55	NG372	imm+	sp+	.06±.01	.15±.00	.25	.23±.02	.16
42	NG205	imm+	sp+	.11±.01	.20±.01	.26	.13±.01	.24±.01
42	NG205	imm-	sp+	.35±.01	.34±.00	.44	.31±.04	.30±.05
42	NG205	imm+	sp-	.24±.02	.40±.02	.31	.32±.03	.36±.07
42	NG205	imm-	sp-	.86±.02	.89±.03	.54	.66±.05	.54±.01

The above numbers represent the average Immunity Value for each phage strain plus or minus the standard error.

rendering them defective in exo V and endo I activity, respectively. As can be seen there is no additional increase in IVs in the doubly defective hosts. This is more or less expected since several investigators have shown that endo I is not involved in genetic exclusion although it is employed in breakdown of superinfecting DNA in the periplasmic space (Fielding and Lunt, 1970; Anderson and Eigner, 1971; Anderson et al., 1971; Silverstein and Goldberg, 1976b).

The effect of a mutated gene 2/61 (pilot protein) on genetic exclusion in an exo V- host

One can speculate that the imm g, alters or removes a protective protein from superinfecting phage DNA thereby exposing it to exoV degradation. Such a hypothetical protein is referred to as a pilot protein (Goldberg, 1983). It follows from this speculation that the level of exclusion seen in an exo V- host superinfected with phage DNA having a defective pilot protein would approximate the level of exclusion seen in the same host superinfected with protected DNA. In other words the presence or absence of a functional pilot protein would be expected to make no difference in the level of exclusion in an exo V- host.

Goldberg (1983) maintains that gene 2/61 is the T4 pilot protein. Silverstein and he (1976a) developed a method to grow gene 2 amber mutants in Su- hosts yielding

Table 3-5: Genetic Exclusion of 2.Su- Superinfecting Phage DNA by Phage T4 imm+/-, sp +/- Strains in E. coli DE828 (Exo V-)

T4 gene	T4 mutant			E. coli host DE828(594,recD-)
55	NG372	imm+	sp+	.18±.05
42	NG205	imm+	sp+	.20±.03
42	NG205	imm-	sp+	.41±.01
42	NG205	imm+	sp-	.45±.05
42	NG205	imm-	sp-	.85±.05

The above numbers represent the average Immunity Value for each phage strain plus or minus the standard error.

phage (2.Su-) that will only form plaque on exo V- hosts. By contrast, DNA is rapidly degraded in exoV+ E. coli. Reasoning that 2.Su- phage would be excluded at the same rate as phage possessing an intact pilot protein in an exo V- host, I performed the standard exclusion assay using 2.Su- as the secondarily infecting phage. By comparing the results in Table 3-5 with those in Table 3-4 for the same host/phage combinations, it can be seen that the IVs are essentially the same, supporting the above prediction. [The alternate experiment where 2.Su- DNA superinfects an exo V+ host would not informative since 2.Su- DNA is rapidly degraded in the host cytoplasm (Silverstein and Goldberg, 1976b).]

Exonuclease III involvement in genetic exclusion

E. coli, also produces an enzyme that catalyzes the 3' to 5' stepwise removal of mononucleotides from double-stranded DNA having an exposed 3'-OH group (Weiss, 1976). The enzyme, a product of the xth gene, has been classified as exonuclease III (exo III).

It seemed reasonable that this enzyme might be involved in the exclusion of superinfecting phage T1 DNA. The results (Table 3-6) of the standard exclusion assay performed in an xth mutant and its isogenic parental strain indicate that there is a decreased exclusion ability (increased IVs) of the primarily infecting phage in the exo

III defective mutant. Therefore, exo III is involved in the exclusion process but its effect does not appear to be linked to the presence or absence of the imm or sp gene products.

Plaque forming ability of 2.Su- phage on various E. coli hosts

As seen in previous experiments exo III and V are involved in the exclusion process while endo I is not. 2.Su- phage are unable to form plaques on exo V+ hosts presumably because the presence of a defective pilot protein (gene 2/64) allows degradation of the 2.Su- DNA by exo V. The pilot protein must therefore protect the 3' end(s) of the infecting T1 chromosome. Since exo V is involved in exclusion and exo V- hosts will titer 2.Su- phage then by analogy it seems possible that exo III hosts may also titer 2.Su- phage. Currently there are no reports in the literature concerning the ability of 2.Su- phage to grow on exo III- (xth mutant) hosts.

The data in Table 3-7 reveals that 2.Su- phage form plaques exclusively on exo V defective hosts, as seen by Silverstein and Goldberg (1976a). They do not form plaques on a strain deficient in exo III activity alone. This implies that the pilot protein effect renders the 2.Su- phage DNA sensitive to exo V activity specifically. The results imply that exo III acting alone or in concert with

Table 3-6: Genetic Exclusion by Phage 11 imm+/-, sp +/- Strains in Various E. coli Hosts (Exonuclease III +/-).

gene	11 mutant	E. coli hosts KL16(xth+)	BW9101(xth-)
55	NG372 imm+ sp+	.03+.01	.10+.00
42	NG295 imm+ sp+	.06+.00	.19+.01
42	NG295 imm- sp+	.32+.01	.47+.01
42	NG295 imm+ sp-	.20+.00	.25+.00
42	NG295 imm- sp-	.75+.03	.83+.09

The above numbers represent the average immunity value for each bacteriophage strain plus or minus the standard error.

a variety of other host enzymes present is unable to degrade 2.Su- phage DNA. The results also indicate that the presence of an additional sbcB mutation does not suppress the effect of the exo V- mutation in its ability to allow growth of 2.Su- phage (Table 3-7, host JC7623 and CES201).

Additionally, the presence of an amber suppressor in the host has no effect on its ability to allow 2.Su- phage to form plaques (Table 3-7, hosts CR63 and JC7623). This indicates that the 2.Su- defect is a structural one (i.e. a pilot protein) rather than a defective product resulting from expression of the infecting parental genome.

The Spackle Gene

As reviewed in Chapter I, the first spackle (sp) mutant was isolated by Emrick (1968) as a suppressor of an e (lysozyme deficient) mutant. The spackle gene product was later shown to provide resistance to lysis from without and was subsequently implicated in promoting genetic exclusion. Emrick (1968) mapped the gene between genes 58/61 and 11, but her results gave only an approximate location. In any particular T1 genetic map, sp might be found in any one of three locations varying by as much as 5kbp on the restriction map. Lau and Berlin (1979) have shown a functional interaction between sp and [illegible] exclusion between sp+ and sp- [illegible]

Table 3-7: Plaque Forming Ability of Bacteriophage T1 (2.Su-) on Various E. coli Strains with Wildtype and Defective Exonucleases III and V, Endonuclease I and Amber Suppressors at 37oC.

E. coli strain	Endo I	Exo III	Su V	T1 strain wildtype	2.Su
S/6	+	+	+	+	-
CR63	+	+	+	+	-
594	+	+	+	+	-
DE828	+	+	-	+	+
JC7623	-	+	-	+	+
CES201	-	+	-	+	-
KL16	+	+	+	+	-
BW9101	+	-	+	+	

+ indicates the presence of the specified endonuclease, exonuclease or suppressor in the E. coli strain shown to the left, - indicates absence of same. In the columns under T1 strains, + indicates growth in the E. coli strain indicated, - indicates no growth.

AD-A186 463 GENETIC EXCLUSION IN BACTERIOPHAGE T4(U) AIR FORCE INST 2/2
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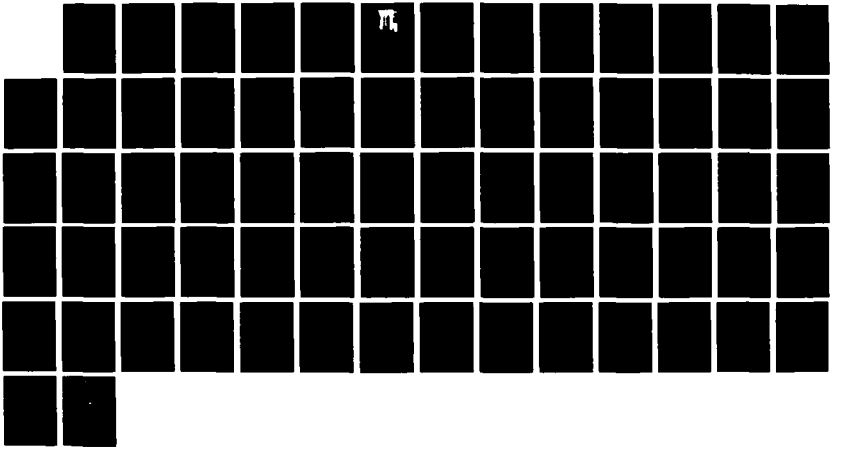
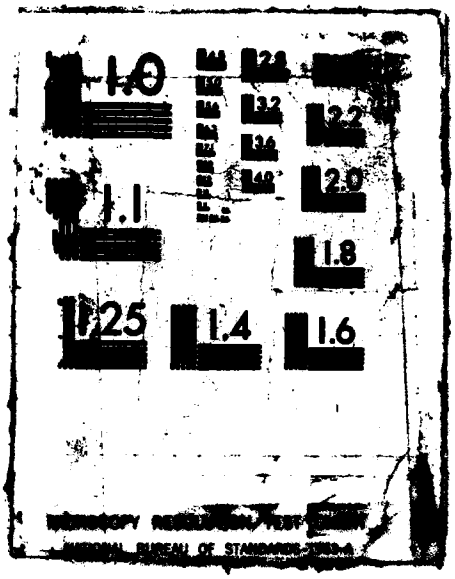
UNCLASSIFIED F/G 6/2 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
3.2
3.6
4.0

about its mechanism of action with respect to the exclusion phenotype. The size of the sp gp is also unknown.

The location of spackle

To more precisely define the location of the sp gene, I obtained a clone (pBSK101) containing a 3.34 kbp EcoRI fragment that extends from 21.155 to 24.495 on the T4 restriction map (see Fig. 3-7) (Kutter and Ruger, 1985). I then performed the standard exclusion assay on an E. coli host containing pBSK101 using two sp- mutants as the primary infecting phage. The success of this assay depends on the ability of the cloned sp gene to express the sp gp at levels sufficient to complement the primarily infecting sp mutant phage. In control experiments I used E. coli without plasmid and the same cells with a non-recombinant plasmid (pBR325), the original cloning vector. Two phage strains were used that were defective in sp, one of them containing an additional mutation in the imm gene. As control phages, I used one strain that was sp+ and imm+ and one that was sp+ imm-. If the sp gene was present in the plasmid and expressing the sp gp should complement the sp- primary infecting phage. This would result in a lower immunity value (i.e. greater exclusion) than in cells without the cloned sp gene. By contrast, exclusion by the sp+ imm+ and sp+ imm- primary phages should change little

when assayed in the host containing the sp- recombinant versus non-recombinant plasmids.

The results obtained with plasmid pBSK101 are presented in Table 3-8. As can be seen from the data, the pBSK101 recombinant plasmid complemented both the sp- imm+ and sp- imm- primarily infecting phage to a level that provided 52% and 62%, respectively, recovery of the sp+ phenotype. (As I considered the host cell with the non-recombinant plasmid, pBR325, to be the most appropriate control, I used its values in all calculations.) This shows that the exclusion assay is an effective method to screen for sp gene activity and that the sp gene is located on pBSK101. To further refine the location of sp, I subcloned smaller DNA fragments of the T4 fragment in pBSK101 according to the scheme in Fig. 2-1. The standard exclusion assay for complementation was performed on each of the subclones in order to locate the sp gene more precisely. The results of these assays (Table 3-8) narrowed down the spackle gene's position to a location contained all or in large part between 21.647 and 22.011 kbp on the T4 restriction map. This fragment is present on pBHE3 which yields approximately 50% recovery (via complementation) of the sp+ phenotype in the standard exclusion assay at 37 ^{o}C.

Table 3-8: Complementation of T4 sp- by Various Recombinant Plasmids

E. coli Strain	Plasmid	sp- --> sp+	sp- imm- --> sp+ imm-
K803	----	0	0
"	pBR325	0	0
"	pBSK101	52	62
"	pPP1	14	-17
"	pBB1	121	68
"	pBHE3	53	43

The values above indicate the percent recovery of the sp+ phenotype measured by the change in IVs assayed in the indicated plasmid bearing cells when infected with an sp- or sp- imm- phage as compared to the IVs of the controls (See Chapter 2 for further explanation). The designation sp- --> sp+ indicates that a sp- primary phage was used to infect various *E. coli* constructs, some containing the sp+ recombinant plasmid, and some not. The designation sp- imm- --> sp+ imm- indicates that a sp- imm- primary phage was used to infect the various *E. coli* constructs. pBR325 is the non-recombinant parental plasmid control. See Fig. 3-7 for the map location of the T4-DNA inserted into each of the designated recombinant plasmids.

The sequence of this region has been partially determined by two different laboratories (Fujisawa, Yonesaki and Minagawa, 1986; M. Nakanishi, 1987, personal communication). However, there are numerous discrepancies between the sequences from the two labs. This dilemma is currently being reconciled (H. E. Selick, 1987, personal communication).

The size of the spackle gene product

To determine the size of the sp gp, I subcloned the 367 bp T4 fragment contained in pBHE3 into an expression vector plasmid with the intent of overexpressing the sp gp and determining its molecular weight. The 367 bp fragment was inserted into the IPTG-inducible expression vector, pUC18, yielding the recombinant plasmid pJO11. As the direction of transcription of the sp region is known (Kutter and Ruger, 1985), the sp containing fragment with HindIII and BglII restriction endonuclease site homologous ends was force cloned into pUC18 at the HindIII and BamHI restriction sites to achieve the proper transcriptional orientation. In conjunction with R. P. McCreary, the molecular weights of proteins from whole cell lysates were determined by SDS-PAGE using the discontinuous buffer method of Laemmli (1970). A protein of approximately 15 kDa appeared in the induced sp (E. coli DH5:pJO11) containing cells that was not present in the controls

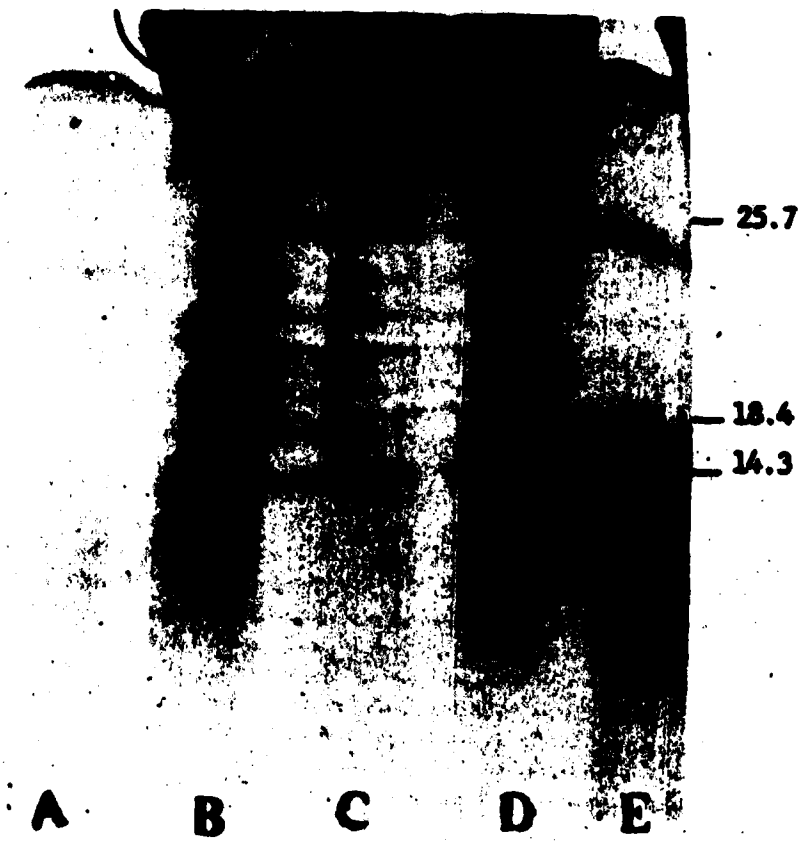


Fig. 3-5: The above photograph is of an SDS-PAGE gel of whole cell lysates stained for proteins. Lanes A and E are molecular weight markers. Lane B is the lysate of the host cell, E. coli DH5. Lane C is the lysate of the host cell plus parental expression vector without the T4 DNA insert, pUC18, grown under inducing conditions. Lane D is a the lysate of the host cell bearing a recombinant plasmid of pUC18 with a 367 bp T4-DNA insert grown under induced conditions. The SDS-PAGE was conducted as described in Chapter 2.

(E. coli DH5 or E. coli DH5:pUC18) (Fig. 3-5). I interpret this as evidence for the molecular weight of the sp protein being approximately 15 kDa.

Coincidently, this finding indicates that the 367 bp sp containing fragment has only one (or most of one) open reading frame (ORF). If all 367 bps are used to encode the primary structure of a protein it would have an estimated molecular weight of about 13.4 kDa. This agrees nicely with our molecular weight determination of the protein expressed by the sp containing fragment. This agreement also argues against the existence of additional expressed ORFs on the 367 bp fragment.

Genetic exclusion of a T4 mutant (5ts1) in gene 5

The mechanism of gp sp action in genetic exclusion is not known, but there are some revealing insights into sp gp's role in resisting lysis from without and lysis from within. Gene product 5, a component of the central base plug, acts as a lysozyme that can cause host cell lysis at a sufficiently high phage multiplicity of infection (Kao and McClain, 1980a; Nakagawa et al., 1985). Gp sp has been shown genetically to interact with gp 5 and presumably to inactivate its lysozyme function (Kao and McClain, 1980b). It has been hypothesized that gp 5 aids penetration of the tail tube during infection by the enzymatic digestion of the murein layer of the host cell wall (Kao and McClain,

1980b). This suggests that gp sp may also contribute to genetic exclusion by inhibiting the ability of the superinfecting phage to properly inject, possibly leading to degradation of the superinfecting DNA in the periplasmic space as previously described. To investigate this idea I obtained a unique gene 5 mutant of T4 (5ts1) isolated by Kao and McClain (1980b) that has a temperature sensitive defect which prevents interaction with gp sp. If 5ts1 phage are not affected by gp sp in exclusion then the prediction is that the IVs of the sp+ primarily infected phage using 5ts1 as the superinfecting phage should closely approximate the values found when sp- is the primary infecting phage. It also follows that the IVs of sp+ primarily infected cells superinfected with 5ts1 would increase toward the IVs of sp- primarily infected cells superinfected with a wildtype gene 5 phage.

The results in Table 3-9 confirm the above predictions. When sp+ phage were used in the primary infection the IVs increased considerably when superinfection was with 5ts1 phage compared with 5+ phage. They approximate the IVs of the sp- primarily infected cells superinfected with gene 5+ phage. Also the IVs obtained with sp+ phage infected cells are not significantly different from the values of the sp- infected cells when both are superinfected with 5ts1. This

indicates, in another way, that the effect is due to an interaction between gp 5 and sp gp and that it is not due to a difference in genetic backgound between 5+ and 5ts1. Collectively these observations can be explained by reasoning that the lysozyme specified by 5ts1 is not susceptible to inactivation by sp gp. Therefore, the infection is not blocked by sp gp. This results in an increased IV when 5ts1 phage are used to superinfect sp+ primarily infected cells. The increased IV is the same as that obtained with sp- primarily infecting phage. These results provide insight into the mechanism of sp gp mediated genetic exclusion in normal infections. Apparently gp sp interacts with gp 5, the lysozyme of the phage's baseplate cental plug, interfering with its ability to aid in tail tube penetration. The interaction thus contributes to the exclusion of the secondarily infecting phage's DNA from the progeny.

Efficiency of plating of phages T4D+ and T4(5ts1) on induced DH5:pJO11.

To further examine sp gp action in an environment isolated from the effects of the remainder to the phage genome, I conducted efficiency of plating (EOP) experiments comparing T4D+ and 5ts1 on three E. coli hosts: E. coli DH5 alone, DH5 containing the plasmid pUC18 (non-recombinant) and on DH5 containing plasmid pJO11 (a recombinant of pUC18

Table 3-9: Genetic Exclusion of Phage T4 (5ts1) by Primarily Infecting Phage T4 imm+/-, sp +/- Strains in *E. coli* Host S/6.

T4 Primary Infecting Phage		Superinfecting Phage	
gene	mutant	5+	5ts1
55	NG372 imm+ sp+	.08±.01	.36±.07
42	NG205 imm+ sp+	.04±.01	.22±.03
42	NG205 imm+ sp-	.26±.03	.31±.10
42	NG205 imm- sp+	.47±.03	.64±.05
42	NG205 imm- sp-	.70±.06	.73±.04

The above numbers represent the average Immunity Value determined by the standard exclusion assay at 37oC for each phage strain plus or minus the standard error.

plus the fragment of T4's chromosome containing the sp gene). The experiments were conducted at temperatures can be seen in Table 3-10, T4D+ is efficiently excluded by DH5:pJO11, especially at the lower temperatures. This alone provides proof that gp sp has the capability to exclude phage and supports the body of data implicating the sp gene's role in genetic exclusion.

As expected, 5ts1 (Table 3-11) is excluded considerably less efficiently by DH5:pJO11 to T4D+. This confirms the ability of 5ts1 to penetrate the sp barrier and supports the findings of the genetic exclusion assays in which the host cells were primarily infected by sp+ phage and 5ts1 was the secondarily infecting phage (Table 3-9).

Efficiency of plating of phages T2 and T6 on induced DH5:pJO11.

Since phages T2 and T6 are closely related to T4, it is of interest to explore the ability of T4 gp sp to exclude T2 and T6. T4 has been shown by several investigators using different techniques (reviewed in Birge, 1981) to be more closely related to T2 than T6, so one would expect interspecific exclusion by T4 sp gp to be more effective against T2 than T6. The titers of T2 on DH5:pJO11 are shown in Table 3-12 and the titers of T6 on DH5:pJO11 are shown in Table 3-13. These results support

Table 3-10: Efficiency of Plating of Wildtype Phage T4 on DH5:pJO11

Host	Plasmid	Incubation Temperature, °C 22	28	37	43
DH5	---	134±10	139±1	162±8	175±1
DH5	pUC18	141±7	139±14	167±12	157±6
DH5	pJO11(sp+)	7±1	19±2	94±3	107±2

The above values represent the number of plaques ± standard error. An equal aliquot of a wildtype phage T4 was plated on each specified E. coli host/plasmid. The plates were incubated at the indicated temperature.

Table 3-11: Efficiency of Plating of a Phage T4 Mutant (5ts1) on DH5:pJO11

Host	Plasmid	Incubation Temperature, °C 22	28	37
DH5	---	94±3	89±3	107±3
DH5	pUC18	102±7	112±1	108±6
DH5	pJO11	78±5	74±1	119±6

The above values represent the number of plaque forming units ± the standard error. An aliquot of a phage T4 mutant (5ts1) was plated on each specified *E. coli* host/plasmid. Then the plates were incubated at the indicated temperature.

Table 3-12: Efficiency of Plating of Phage T2 on DH5:pJO11

Host	Plasmid	Incubation Temperature, oC 22	28	37	43
DH5	---	37±4	36±3	33±5	27±4
DH5	pUC18	56±6	48±2	53±2	47±6
DH5	pJO11	13±2	16±3	11±1	9±2

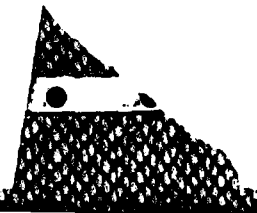
The above values represent the number of plaque forming units ± standard error. An equal aliquot of phage T2 (wildtype) was plated on each specified *E. coli* host/plasmid. Then the plates were incubated at the indicated temperature.

Table 3-13: Efficiency of Plating of Phage T6 on DH5:pJO11

Host	Plasmid	Incubation Temperature, °C 22	28	37	43
DH5	---	114±5	125±1	149±2	124±5
DH5	pUC18	138±9	131±2	127±7	141±5
DH5	pJO11	128±0	132±3	131±5	132±2

The above values represent the number of plaques ± standard error. An equal aliquot of phage T6 (wildtype) was plated on each specified *E. coli* host/plasmid. Then the plates were incubated at the indicated temperature.

Fig: 3-6: Each of the values represented above were obtained by dividing the number of pfu from an equal aliquot of each phage titered on DH5:pJO11(sp+) by the number of pfu on DH5:pUC18 x 100. This yields the percent of pfu able to titer on the sp containing clone. The pfu counts used to calculate the eops on the two hosts are shown on Tables 3-10, 3-11, 3-12 and 3-13. T4(5ts1) was not titered at 430C since it is unable to grow at this temperature.



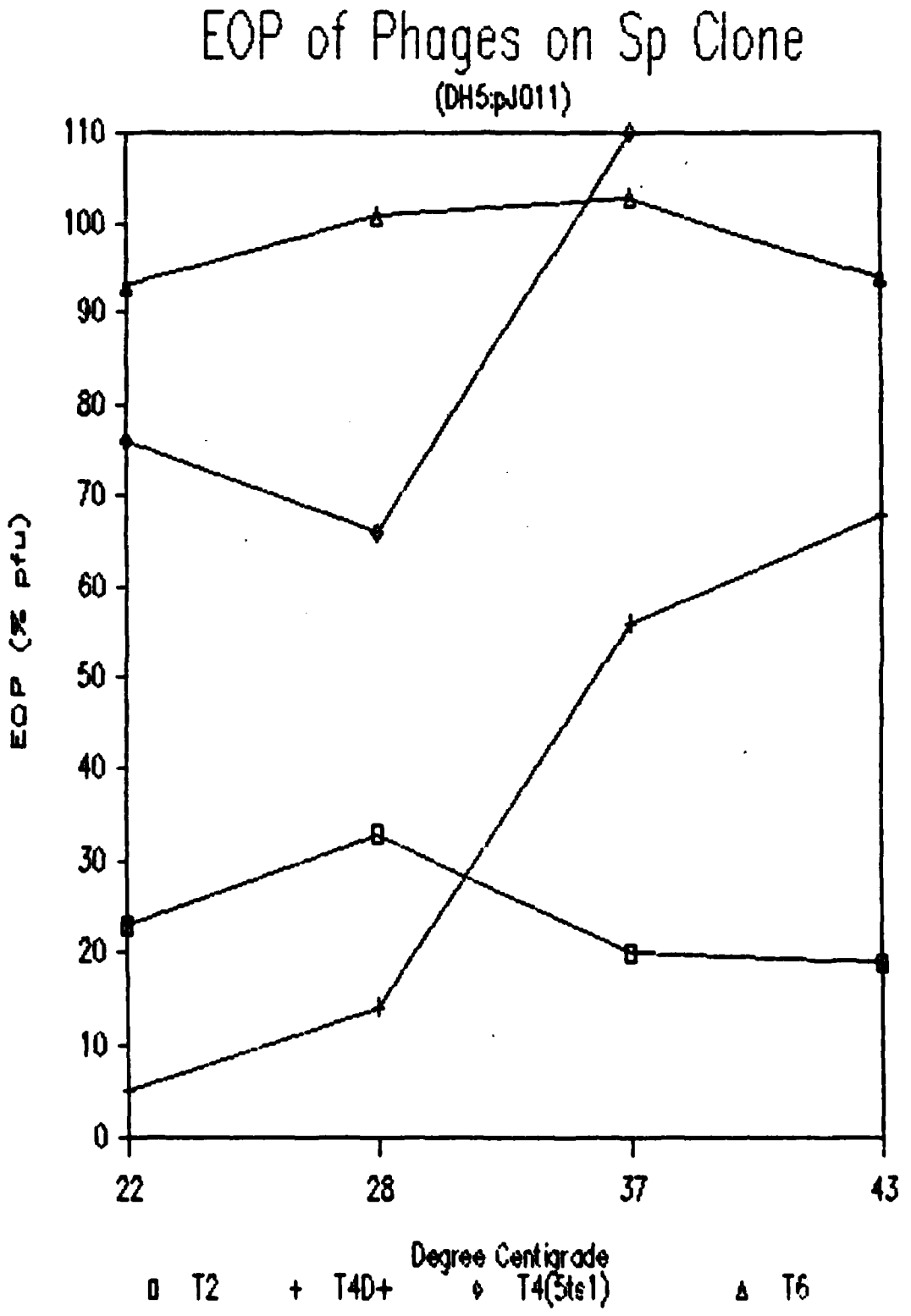

EOP of Phages on Sp Clone
(DH5:pJ011)
EOP (% pfu)
110
100
90
80
70
60
50
40
30
20
10
0
22
28
37
43
Degree Centigrade
T2
T4D+
T4(Sts1)
T6

Fig. 3-6:

the expectation that T2 should be excluded more effectively than T6. For example the T4 gp sp producing clone ranging from 22 °C to 43 °C under inducing conditions. As (DH5:pJO11) excludes 86% of T4, 67% of T2 and 0% of T6 at 28 °C compared to the ability of the same phages to titer on the same E. coli host containing the non-recombinant parental plasmid (DH5:pUC18).

A comparison of the exclusion capability of DH5:pJO11, presumably by the T4 sp gp, against phages T4D+, T4D(5ts1), T2 and T6 is shown in Fig. 3-6.

The Relationship of Genes Spackle and 40.

My previous results locate the spackle gene between 21.647 and 22.014 on the T4 restriction map (Kutter and Ruger, 1985). This location appears to be the same region on the T4 restriction map (Figs. 1-2, 3-7) (Kutter and Ruger, 1985) as gene 40. The molecular weight of gp 40 has been reported to be 14 kDa (Black, 1974) or 18 kDa (Laemmli, 1970). Considering the size of the sp gp it seems unlikely that the region previously allocated to gene 40 between genes 41 and uvsx (about 350 bps) could contain two ORFs each encoding for a protein product of approximately 15 kDa. The location of gene 40 is supported by a considerable amount of genetic and molecular data (Black, 1974; Black, Zachary and Manne, 1981; Brown and Eiserling, 1979 a and b; Hsaio and Black, 1978b). Since I

Table 3-14: Efficiency of Plating of ocL84(40) on E. coli Strains.

Host plasmid	Phage/incubation temperature °C		
	T4D/45	ocL84/30	ocL84/45
K803 pBR325	+	244	0
K803 pBHE3 (1)	+	348	55
(3)	+	307	48

The above values represent the number of plaque forming units from an equal aliquot of a stock of a phage T4 mutant (ocL84) plated on E. coli hosts and then incubated at the indicated temperatures. The + indicates that wildtype T4D forms plaques on the hosts at 45oC. The numbers in parenthesis after pBHE3, (1) and (3), represent two different transformants containing the same recombinant plasmid.

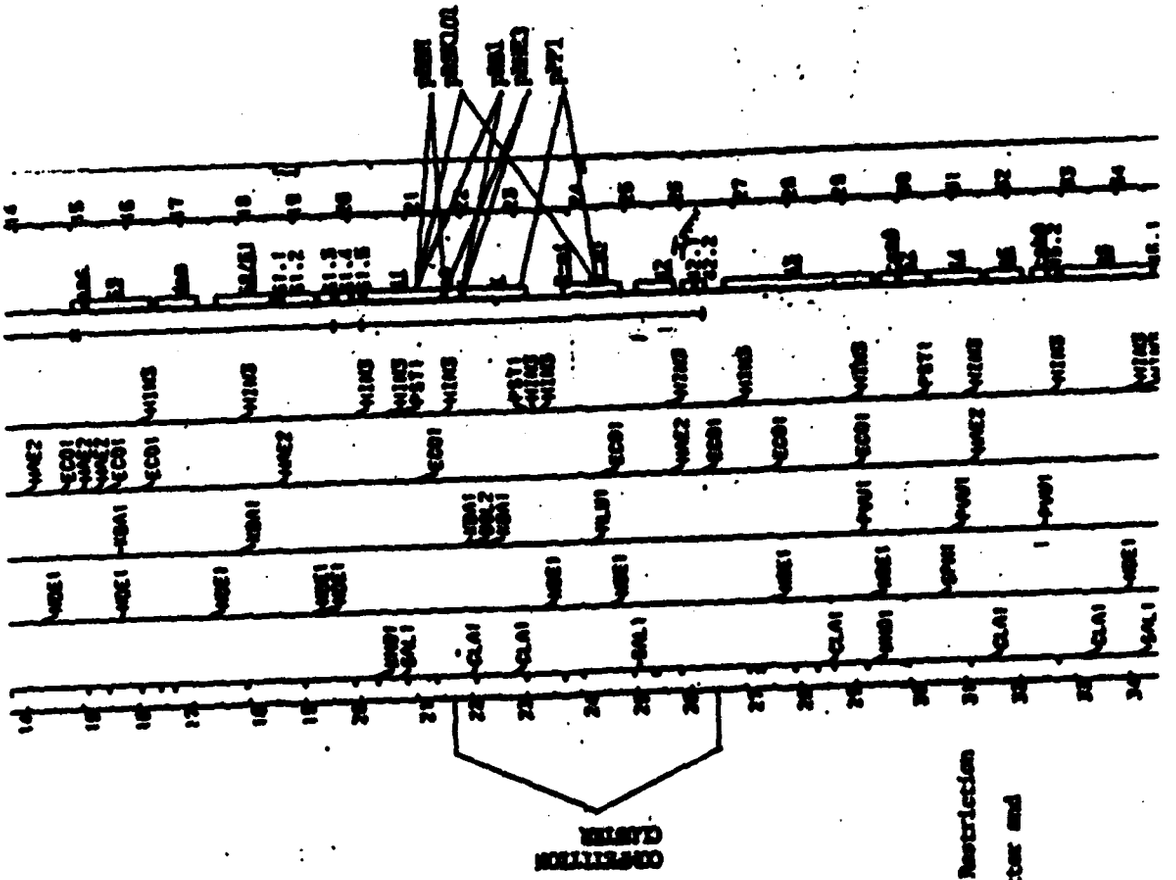


Fig. 3-7: Phage λ Restriction map. Taken from Kaiser and Hogness, 1983.

had previously shown the location of spackle to be in the same region, the most conservative explanation for these apparently contradictory facts is that gene spackle and gene 40 are the same gene producing one gene product that accounts for both phenotypes. I therefore carried out experiments to support or disprove this explanation.

Efficiency of plating of a gene 40 mutant on a sp clone

If genes sp and 40 are indeed the same, then it follows that a clone that has shown sp activity should also display gene 40 activity. It would be expected that the EOP of a gene 40 mutant (ocL84) would increase when titered under restrictive conditions on sp containing host cells (K803:pBHE3), when compared to the EOP on host cells containing the non-recombinant parental plasmid (K803:pBR325). The restrictive conditions are infection of a su- strain at high temperature, since gene 40 is essential for phage growth only at high temperatures. The results are presented in Table 3-14. An increase in EOP of ocL84 is observed when ocL84 is titered on two separate clones containing a 367 bp insert of T4 DNA that had previously shown sp activity (refer to Table 3-8 for spackle complementation and Fig. 2-1 for plasmid pBHE3 genealogy). Both of the clones allow the gene 40 mutant to form approximately 16% of the plaques under restrictive conditions that are formed under permissive conditions.

This implies that the sp containing clones are able to express partial gene 40 function.

Spot tests using a gene 40 mutant on various E. coli hosts

Mattson et al. (1977), Mattson, Van Houwe and Epstein (1983), and Velten and Abelson (1990) have described efficient methods to assay plasmid-bearing host cells for an insert of DNA containing a particular gene. This technique involves spotting a suspension of a phage mutant defective in the gene in question on a spot of the plasmid bearing host cells over a lawn of restrictive cells. This spot test method was used to test the recombinant plasmids having the T4 DNA inserts which include the sp gene or a region near this gene (see Fig. 3-7 for location of the plasmid inserts on the phage T4 map and Fig. 2-1 for the plasmid genealogy). A photograph of the results of the spot tests is shown in Fig. 3-8. In Fig. 3-8 clearing is represented by a dark spot due to the photographic technique used. A complete clearing of the spot indicates either a high level of complementation or a high level of cross-reactivation (also known as marker rescue). The difference between the two explanations for clearing is as follows. Complementation occurs when the product of the gene being tested by the phage mutant is provided by the inserted DNA contained in the recombinant plasmid. A high level of complementation allows bursts of sufficient siz

from the host cells containing the complementing plasmid to provide enough mutant phage to adsorb to and kill the cells of the indicator lawn. This causes a clearing of the indicator lawn. Often a low level of complementation occurs providing only enough progeny phage to partially kill the indicator lawn cells in the spot. This gives rise to a partial clearing of the spot (hazy spot). By contrast, in cross reactivation or marker rescue the protein product of the gene inserted in the plasmid is not expressed but the DNA is homologous to the mutated tester phage gene. This allows recombination to occur, restoring a wildtype gene in place of the mutated allele in the tester phage (i.e. the marker gene in the phage is said to be rescued or cross reactivated). The resultant bursts then contain phage with a wildtype gene which enables the progeny to carry out a normal cycle of infection resulting in plaque formation on the indicator lawn and hence clearing in the spot. When the frequency of recombination (cross-reactivation or marker rescue) is high enough the entire spot clears; lower levels of recombination are indicated by plaque formation rates within the spot that are greater than those of the controls.

The photograph in Fig.3-3 shows a significant degree of clearing in the spots designated as C, D, H, K and L. On spots C and D the cI.84(gene 10) was spotted on a sp

Fig. 3-5: The above photograph is of an SDS-PAGE gel of whole cell lysates stained for proteins. Lanes A and E are molecular weight markers. The relevant markers are labelled in kDa along the right side of lane E. Lane B is the lysate of the host cell, E. coli DH5. Lane C is the lysate of the host cell plus parental expression vector without the T4 DNA insert, pUC18, grown under inducing conditions. Lane D is a the lysate of the host cell bearing a recombinant plasmid of pUC18 with a 367 bp T4-DNA insert grown under induced conditions. The SDS-PAGE was conducted as described in Chapter 2.

containing expression vector clone (pJO11-induced to expression) showing complete clearing. This is probably due to complementation since both clones were in an induced state. Spot K shows the result in the K803:pBB1 host which carries an 837 bp fragment of T4 DNA containing sp. Spot L shows the effect on a clone (K803:pBHE3) having a 367 bp sp containing insert. The indications in spots K and L are most likely the result of cross reactivation. Spot H shows a degree of complementation to ocL84(40) that will be addressed in Chapter 4. Spot G shows a low level of marker rescue of the tester phage that is not readily explainable. It will be treated as background for the purpose of this study. The contol spots are listed in the legend to the figure. The above spot tests give strong preliminary indications that plasmids pBB1, pBHE3 and pJO11 contain all or a large portion of gene 40 as well as the sp gene.

Quantitative complementation of a gene 40 mutant

To further verify the results seen in the spot test experiments and measure the level of complementaion provided by each of the plasmids, I performed quantitative complementation experiments with the mutant ocL84(gene 40) on the previously described clones by the method of Mattson et al. (1977). The findings (Table 3-15) clearly show complementation by several plasmids and further verify the results of the spot tests. As expected pJO11, the

expression vector sp containing plasmid, in the induced state complements ocL84(40) most effectively showing an 313.8 fold increase in burst size over the control (host with parental plasmid). Both pBB1 and pBHE3 complement but to a lesser degree yielding an increase in burst size of 9 and 5 respectively. Additionally the results in pBB1 and pBHE3 add credibility to the standard exclusion assays done on these clones when screening for the presence of the sp gene, since the assay relies on the production of the sp gp from the insert even though it is in a non-expression vector (pBR325 parent). Please note that G240:pPP1 also complemented ocL84 at a low level which will be discussed in Chapter 4.

Quantitative marker rescue of a gene 40 mutant

Using the method of Mattson et al. (1977) and Mattson et al. (1983) I performed quantitative marker rescue experiments on the clones derived from the presumed sp containing area of the T4 genome. The results in Table 3-16 clearly show the clones determined to contain sp (pJ011, pBHE3, pBB1) also contain at least part of gene 40 by the criterion of marker resue. pBB1 gave the highest level of marker rescue with a frequency 50 fold greater than the control. These results demonstrate that the sp containing clones also contain DNA homologous to gene 40 which can allow cross-reactivation of the gene 40 mutant.

Table 3-15: Complementation of Gene 40 Mutant (ocL84)

E. coli Strain	Plasmid	Burst Size	Factor of Increase over Control
DH5	---	6.4×10^{-2}	-
DH5	pUC18	5.8×10^{-2}	1.0
DH5	pJO11	18.2	313.8
K803	---	5.9×10^{-2}	-
K803	pBR325	2.0×10^{-2}	1.0
K803	pBHE3	1.0×10^{-1}	5.0
K803	pBB1	1.8×10^{-1}	9.0
G240	---	9.8×10^{-3}	-
G240	pBR325	6.7×10^{-3}	1.0
G240	pPP1	2.3×10^{-2}	3.4
G240	pBBH	9.3×10^{-3}	1.4

The values under the column entitled "Burst Size" are the burst sizes of an infection with phage ocL84(gene 40) in the indicated hosts. The assays quantitate the degree of complementation of the defect in ocL84(gene 40) by plasmid gene products. Those values under the column entitled "Factor of Increase over Control" show the relative increase in burst size as compared to the host cell with the parental non-recombinant plasmid. Plasmids pUC18 and pBR325 are the parental plasmids for the recombinant plasmids being assayed. The assays were conducted as described in Chapter 2.

Table 3-16: Marker Rescue of Gene 40 Mutant (ocL84)

E. coli Strain	Plasmid	Marker Rescue Frequency	Factor of Increase over Control
DH5	---	2.5×10^{-4}	-
DH5	pUC18	1.7×10^{-4}	1.0
DH5	pJO11	2.3×10^{-3}	13.5
K803	---	1.1×10^{-4}	-
K803	pBR325	9.0×10^{-5}	1.0
K803	pBHE3	3.4×10^{-3}	37.8
K803	pBB1	4.5×10^{-3}	50.0
G240	---	2.0×10^{-4}	-
G240	pBR325	1.1×10^{-4}	1.0
G240	pPP1	1.3×10^{-4}	1.2
G240	pBBH	2.0×10^{-3}	18.0

The values under column "Marker Rescue Frequency" are the frequencies of wildtype progeny from an infection of ocL84(gene 40) in each of the respective hosts. The values under column "Factor of Increase over Control" show the relative increase in occurence of the wildtype gene 40 marker in the progeny compared to the control. The values of the host cell bearing the parental plasmid were used in the calculations as the background marker rescue frequency. Therefore they were scored as having a factor of 1.0. Plasmids pUC18 and pBR325 are the parental plasmids for the recombinants being assayed. The assay quantitates the amount of cross-reactivation (marker rescue) via homologous recombination occuring during the infection between plasmid and phage. The assays were conducted as described in Chapter 2.

Standard genetic exclusion assay using gene 40 mutants

One could argue on the basis of the EOP, complementation and marker rescue data using the mutant ocL84(gene 40) that there are two ORFs present on the recombinant plasmids, one encoding the sp gp and the other gp 40. I consider this possibility unlikely, since it is contradicted by the small sizes of the inserts which are insufficient to encode the measured gps of both sp and 40. However, this possibility is easily checked. If genes 40 and sp are the same gene, mutants defective in gene 40 should also show a defective genetic exclusion phenotype and probably to the approximate levels of the known sp- mutant. I therefore used three mutants of gene 40 (Hsaio and Black, 1978b), one defective at the amino encoding end (amB49) and two at the carboxy encoding end (amA104 and ocL84), to perform the standard exclusion assay. I then compared their IVs to those of the contol phage, one sp+ and the other sp-. The results are shown in Table 3-17. The IVs of the gene 40 mutants closely approach or exceed the sp- phage. This clearly indicates a defective genetic exclusion phenotype for the gene 40 mutants and provides compelling evidence that genes 40 and sp are one and the same. Interestingly, one of the gene 40 mutants (amA104) whose site of defect maps to the carboxy end of the gene exhibited a significantly more defective exclusion

Table 3-17: Genetic Exclusion Phenotype of Three T4D gene 40 Mutants.

T4		IV on *E. coli*
gene	mutant	S/6
42	NG205 imm+ sp+	.07±.01
42	NG205 imm+ sp-	.26±.03
40	ocL84 imm+ sp?	.21±.01
40	amB49 imm+ sp?	.27±.01
40	amA104imm+ sp?	.39±.01

The above values represent the Immunity Value ± standard error.
The values were determined using the standard exclusion assay.

phenotype than the standard sp mutant. On E.coli S/6 at 37°C, this defect accounted for nearly 40% of the exclusion capability of wildtype phage measured under the same conditions.

Plaque forming ability of a Sp mutant at high temperature

Since gene 40 mutants are known to be lethal at high temperature it would seem likely that a sp mutant would behave similiarily assuming that genes sp and 40 are the same. But as Table 3-18 shows, the sp mutant forms plaque at high temperature as efficiently as it does at low tempertures on E. coli host CR63. This confirms the results of Emrick (1968). This observation has implications as to the sp gp's functional organization, as will be discussed in Chapter 4.

Table 3-18: Efficiency of Plating of a Phage T4 Mutant (amNG205, Sp-) on E. coli CR63 at Various Temperatures.

	Incubation Temperature, °C			
Host	22	28	37	43
CR63	154±2	155±16	186±15	169±6

The above values are the numbers of plaques ± the standard error formed when an equal aliquot of phage T4 mutant (amNG205 sp-) was plated on E. coli CR63 and the plates incubated at the indicated temperature.

CHAPTER 4

DISCUSSION

Characterization of the imm-2 mutant phenotype

To aid in the investigation of genetic exclusion, the phenotype of the standard imm- mutant, imm-2, was further characterized. The protein molecular weight findings (Yutsudo, 1979; O'Farrell and Gold, 1973) suggest that imm-2 might be a nonsense mutant since protein bands were missing on SDS-PAGE gels of the protein products of imm-2. However, Vallee and Cornett (1973) had determined that imm-2 was not an amber mutation. As the results in Table 3-1 show, I found that imm-2 is also not a opal nonsense mutant suppressed by the insertion of tryptophan, nor is it an amber or ochre nonsense mutation suppressed by the insertion of lysine or tyrosine. These experiments do not entirely rule out the possibility that imm-2 is a nonsense mutant, since it may require suppression by an amino acid not inserted in the above hosts.

As indicated in Table 3-2 and the corresponding lines in Fig. 3-2 a comparison of the imm mutant's Immunity Values (IVs) with those of the imm+ wildtype over a range of temperatures from 25 °C to 42 °C shows that imm-2 never attains the wildtype IV. This indicates it is neither cold nor temperature sensitive.

Based on the above results imm-2 is not obviously suppressible, not temperature sensitive and not cold sensitive. As a result, all subsequent experiments using imm-2 were designed to take these characteristics into account.

The imm gene shares an out-of-phase overlap with gene 42

I have obtained genetic data which provides strong support for the hypothesis that the imm gene overlaps substantially with gene 42. Much of the discussion concerning the overlap between the imm gene and gene 42 has been presented in Chapter 3 with the experimental results, so only a brief review of the findings is presented here. I assayed 30 amber mutants defective in genes 42 and 43 (the two neighbors bounding the imm gene) and found 7 mutants in gene 42 that displayed a significantly defective exclusion phenotype (Table 3-2, Fig. 3-2). Six of the seven map to the amino terminal one-half of gene 42. (The seventh mutation site has not been mapped.) Since other amber mutants upstream of the most distal exclusion defective gene 42 mutant show a wildtype exclusion phenotype, I concluded that the imm gene and gene 42 substantially overlap in an out of phase orientation (Fig. 3-1).

amNG411 is not a double mutant

Phage amNG411(42) showed a very strong exclusion defective phenotype. To counter the argument that it may contain two mutations (one in gene 42 and the other in the imm or sp gene), I determined the exclusion phenotype of amNG411 phage reverted for the conditional lethal gene 42 phenotype. All of the revertants more closely resembled wildtype in their exclusion than either the amNG411 parent or the imm-2 control (Table 3-3, Fig. 3-3). This indicates that amNG411 is not a double mutant, and that gene 42 is somehow involved in the exclusion process.

One might be tempted to postulate that gene 42 has a dual function, one of which is to promote genetic exclusion. There are two lines of evidence that argue against this idea. First, if that were true then one would expect all amber mutants whose defect is upstream of the most distal exclusion defective gene 42 mutant to also be defective in the exclusion phenotype, but this is not the case (Fig. 3-1). Second, when amNG411 was assayed for its exclusion phenotype in a suppressor host one would reasonably expect the exclusion phenotype to be suppressed also. However this is not the case (Fig. 3-2).

If one assumes that the amber mutations in gene 42 are reverse polarity mutations one might attempt to explain these effects on imm gene function. Presumably in the

comes from the classic work of Bauerle and Margolin (1966). They described a system in the S. typhimurium tryptophan pathway where mutations in a downstream gene had a severe, reverse polar affect on the function of an upstream gene. Mutations in the downstream gene closer to the operator-proximal end had a more severe inhibitory effect on expression of the upstream gene. They further demonstrated that the two gene products in question function in a unique multifunctional enzyme complex. They explained their results by reasoning that as the mutations became more operator-proximal the downstream polypeptide was progressively shorter and therefore less likely to interact positively in the functional complex. This mimicked a defective phenotype in the upstream gene, since its product could only function in the complexed form. An explanation such as this does not seem applicable to the gene *amm*/*l2* relationship. The results in table 3.2 show that the most operator-proximal mutation in gene 12 (*amm*2021) assayed is wildtype for exclusion. When is methylated at [illegible] *amm*C111 is assayed for exclusion it suppresses [illegible] it remains defective for [illegible] hydroxymethylase function (e.g. [illegible] of no work indicating that [illegible] complex.

I conclude that the best explanation of the gene 42 mutant exclusion results and the amNG111 revertant results is that genes imm and 42 significantly overlap in an out of-phase orientation. This would represent the first case of an extensive gene overlap in phage T1. This should have significant evolutionary, ecological and gene regulation implications. [Some short overlaps have been found such as the 1 bp overlap involving a termination and start codon described by Chu et al. (1984)]

An opal codon appears to be involved in exclusion

Preliminary sequence analysis of the imm gene region (Gram, personal communication, 1985), indicates one opal codon in an ORF that extends from the imm gene region well into the structural portion of gene 42. Based on the results of the exclusion assays done in opal(UGA) suppressing hosts (Fig. 4-1), I conclude that the opal codon in the imm gp is normally read-through as some amino acid, but that the preferential insertion of trp at this site [illegible] its function. These results provide further evidence supporting the gene imm/42 overlap hypothesis. They also imply that the larger imm protein seen on SDS PAGE gels is the gp responsible for the exclusion function.

A Multiple overlap is Postulated

To explain the occurrence of [illegible] imm mutants that have been reported as [illegible] independent [illegible]

Chapter 1), I further postulate that the opal codon on the mRNA transcript of the region is normally read-through with less than 100% efficiency. Assuming that genes 42 and imm overlap and that the imm gene actually encodes for two proteins, then a multiple overlapping gene situation would exist within that region. Yutsudo (1979) refers to the sequence that codes for the larger imm protein as gene imm a and the one that codes for the smaller protein as gene imm b. Using this terminology, there would be an out-of-phase overlap between imm a and gene 42 plus an additional in-phase overlap between imm a and imm b. The entirety of imm b would share the first 256 bp with imm a and presumably be terminated within imm a during translation by the embedded opal termination codon. It is commonly held that in-phase overlaps (reviewed in Normark et al, 1983) are a way to produce a pair of proteins that either (i) interact with the same target with their common region while performing different functions via their unique domains or (ii) interact directly to produce a functional complex (van de Hondel, Konings and Shoenmakers, 1975; Smith and Parkinson, 1980). Although no function has been assigned to the imm b gene product, a theoretical argument can be made for its utility based on the continued existence of its opal termination codon. Simply, a genetic element as powerful as a termination codon would not be

selectively maintained in the phage genome in the middle of a gene (i.e. imm a) were it not an advantage. The selective advantage must be due to the function of the protein that the nonsense codon terminates so the region of DNA responsible for the product constitutes a gene (i.e. imm b). There is an example in yeast that sets a precedent for the hypothesized imm a/imm b two protein situation in T4. It seems that the MATa gene cassette that determines mating type in yeast also produces two proteins from the same section of DNA. And the longer protein is produced by the read-through of an embedded opal codon less than 100% of the time (reviewed in Lewin, 1987). The larger protein participates in mating functions, while the smaller protein's function is unknown.

Significance of the Gene imm/42 Gene Overlap

From a gene regulation perspective overlapping genes are of interest because according to Normark et al. (1983), they can have "important regulatory implications both at the level of expression and at the level of protein-protein interaction." Interestingly enough, several lines of evidence show gp 42 to function in a kinetically coupled dNTP-synthesizing complex thought to be attached to the host membrane that works in close association with the gene 13 containing replication complex (reviewed in Mathews and Allen, 1983). Although I know of no evidence directly

connecting the imm gp to this complex, there is considerable evidence indicating that imm also functions at the membrane so there may be some connection.

Normark's group (1983) also suggests that overlapping genes may provide a tool to study the evolution of coding and control sequences. A case has been made for the natural selection of overlapping genes to code for a particular protein quality such as membrane binding. Studies of overlapping genes in phage MS2 (Beremand and Blumenthal, 1979) and in ØX174 (Barrel, Air and Hutchison, 1976) have shown that a +1 shift in reading frame results in the encoding of a very hydrophobic (possibly membrane binding) protein. Interestingly, the sequence data for imm predicts a large hydrophobic amino terminal region (Gram, 1985, personal communication).

Ecological Aspects of the Gene imm/42 Overlap

The possible regulatory connection between imm and gene 42 takes on additional significance when one considers the ecological aspects of their functions. The imm gene by providing immunity to superinfection is, in effect, establishing a territory for the first infecting phage. The infected cell becomes the exclusive resource of the primary infecting phage for use in self propagation. Immunity may thus be an adaptation to intraspecific competition. Gene 42 (dCMP-hydroxymethylase) alters phage DNA in a way that

protects progeny phage from the host (prey) restriction enzymes. Thus gp 42 is an adaptation in a form of interspecific competition.

Another way of looking at the association of the imm gene and gene 42 is that the overlap is part of a regulatory device to prevent expression of gene 42 (essential for phage DNA replication) until after the imm gene is transcribed. This would postpone DNA replication until competing genomes have been excluded by the action of the imm gp thus ensuring that only the initial phage's own DNA is replicated.

Some insights into the molecular mechanism of genetic exclusion

1. Host exonuclease involvement in T4 genetic exclusion.

It is clear from the results presented in Chapter 3 that the primary infecting phage recruits the use of at least two host exonucleases to genetically exclude superinfecting phage. Both exo V and exo III are involved, but they are used in somewhat different strategies.

The role of exo V in exclusion seems to be imm gp dependent; that is, exo V is not a player in the exclusion process unless the primary infecting phage carries a functional imm gene. One interpretation of the results in Table 3-4 is that gp imm interacts with the superinfecting

DNA and exposes the 5' end as a substrate for host exo V action.

It is thought that T4 DNA is protected by the product of gene 2/64, the hypothetical pilot protein. The results in Table 3-5 showing that the presence of a fully functional gp 2/64 is inconsequential in an exo V- host supports the idea that gp imm may interact with the pilot protein altering its protective capacity. With the T4 chromosome unprotected it may become a substrate for exo V nuclease activity.

In addition to the nuclease activity, exo V has been shown to bind to double strand (ds) DNA ends, and then to rapidly move along the DNA, unwinding it. This produces single-strand (ss) loops which the enzyme cuts to release ss fragments (reviewed in Telander-Muskavitch and Linn, 1981). These fragments then become a substrate for additional host DNA degrading enzymes.

Primary phage apparently protect their own DNA from exoV by producing a protein (T4 rec inhibitor) that stoichiometrically inhibits the ATP-dependent exonuclease and DNA-dependent ATPase activities of host exo V (Behme, Lilly and Ebisuzaki, 1976). This effect was shown at 10 min post-infection and was proposed to provide protection for the phage DNA during replication and recombination.

As originally postulated by Kornberg (1974), the function of the pilot protein of the phage nucleoid is to provide specificity and possibly structural help in transferring the phage DNA from the virion into the host cell cytoplasm and to aid in initiation of DNA replication. This concept was most fully developed in phage ØX174 (Jazwinsky, Marco and Kornberg, 1975). If it is true that the T4 pilot protein, gp 2/64, aids in replication, then the interaction of gp imm with the pilot protein of superinfecting phage DNA may profoundly interfere with its replication. The data in Table 3-3 and Fig. 3-3 show that excluded phage aren't replicated to form progeny, but this is a general observation and does not specifically address the question of replication inhibition by an altered pilot protein.

The exoIII participation in T4 genetic exclusion shown in Table 3-6 is not dependent upon the presence of gp imm or gp sp. One might speculate that its activity is regulated by another phage product. Alternately, it may function in a more general fashion by degrading any unprotected DNA in the cytosol. In any case some phage product must have initially been responsible for making the superinfecting ds-DNA susceptible to exo III.

2. The action of gp sp.

As discussed in Chapter 3, Tables 3-9 and 3-11, Fig. 3-6, gp sp seems to act in genetic exclusion by interfering with the lysozyme function of a phage tail central base plug component, gp 5, thereby preventing successful superinfection. In order to accomplish this, I assume that gp sp must either associate with the bacterial cell wall or act at a site exterior of the murein layer. The basis for this assumption is that gp sp must either alter the recognition site of gp 5 action, or directly interact with gp 5 before it reacts with its substrate in the murein layer, or both. Cornett (1974), observed a reduced efficiency of DNA injection by superinfecting phage into cells primarily infected with sp- phage compared to sp+ phage. This observation suggests that the sp gp affects a phage recognition site in the cell envelope. Since DNA injection is triggered by irreversible phage binding to the cell surface it would seem that the gp sp+ does alter an adsorption site. However Cornett's observation seems the reverse of what one might expect. If gp sp+ is acting to exclude superinfecting phage, one would expect cells infected with sp+ phage, not sp- phage, to cause a reduced efficiency of secondary phage injection. I suggest the following explanation for this dilemma based on the notion that an effective defense can counter an offense only if

the opponent can be engaged. Together the above findings suggest that gp sp+ increases the efficacy of exclusion by molecularly highlighting the gp sp+ containing adsorption sites. Presumably, this would decoy the phage to adsorb preferentially at these altered sites. Irreversibe binding would occur, immediately triggering injection. The injection would proceed normally until the tail tube approaches the murein layer where the appropriate counter measure (gp sp's anti-lysozyme activity) is stationed to inactivate gp 5, thereby preventing further tail tube penetration. The DNA might then be extruded into the periplasmic space and degraded by periplasmic enzymes. So despite the fact that gp sp+ increases the efficiency of injection, the net effect is to decrease the chance of a successful superinfection.

One mutant, A104(40) (Fig. 3-17), has a sp- phenotype that is approximately 40% defective in exclusion. This 40% plus the 50% contribution of the imm gene accounts for most, but not all, of the genetic exclusion seen in wildtype T4.

A molecular model of T4 exclusion by gps imm and sp

Upon primary infection, phage T4 begins its life cycle. The early genes are expressed by transcription mediated by unmodified E. coli RNA polymerase. Gps imm and sp are early gene products. They depart the ribosom and

take up their stations within the cell envelope. Based upon the work of Vallee and Cornett (1972, 1973) the imm protein probably takes up a position at the adhesion sites where the cell wall and cytoplasmic membrane are contiguous, as described by Bayer (1968). These adhesion sites have been shown to be the adsorption and injection sites for phage T4, and several other phages, particularly T2 (Bayer, 1970). I suspect that the gp sp joins gp imm at or near these sites. The sp protein presumably further modifies the outer membrane, directly or indirectly, so as to molecularly highlight the adsorption site. As a secondary phage approaches, it is preferentially attracted to the modified site, adsorbs and attempts to inject. But due to the anti-lysozyme affect of gp sp on the incoming gp 5 the injection is blocked in some but not all superinfections. The blockage occurs because of the inability of the tail tube to penetrate the cell wall when the lysozyme is inactivated. The DNA extruded from the successful superinfections now encounters the imm protein. I postulate that gp imm interacts with the chromosome's terminal pilot protein. The interaction results in a number of effects. First, the ds-DNA ends are exposed to exo V degradation and probably unwinding. The unwound single stands are cut into ss fragments that are further degraded by host enzymes such as exo III. Also, the pilot

protein maybe sufficiently altered so as to no longer be able to assist the superinfecting phage in initiating replication.

Together, gps imm and sp genetically exclude superinfecting DNA at the cell wall by interfering with injection, and in the cytoplasm by exposing it to host nuclease degradation and possibly by interfering with initiation of replication. All in all, this appears to be a rather effective intraspecific competition arsenal.

The Competition Cluster

I have previously discussed the role of the imm gene, the sp gene and gene 42 in intraspecific competition. However, an expanded view of this region of the phage T4 chromosome (Fig. 3-7) reveals other genes adjacent to imm and gene 42 which are also involved in phage competition. Bordering gene 42 is Bgt (beta-glucosyltransferase) which acts to glucosylate phage hydroxymethyldeoxycytosine containing DNA. Together, genes 42 and Bgt provide the valuable edge to the predator phage in competition with the E. coli prey by rendering the host endonuclease defenses ineffective against the phage DNA.

Further downstream, I recently defined the location of the sp gene in the region between 21.617 and 22.014 kbp on the T4 restriction map (Kutter and Ruger, 1985) (Table 3-8, Fig. 3-7). I also determined that the molecular weight of

the sp protein is approximately 15 kDa (Fig. 3-8). The sp gene functions most effectively in intraspecific competition at the lower temperatures (Table 3-10, Fig. 3-6), but as can be seen in Table 3-12 and Fig 3-6, it excludes T2 (interspecific competition) very effectively at all temperatures. Phage T6 is essentially unaffected by the exclusion effects of gp sp (Table 3-13, Fig. 3-6). These results also confirm the relatedness of phages T1 and T2. Some characteristic of phage T6, perhaps the gp 5 analog or its adsoprtion site specificity has diverged significantly during the evolutionary separation between phages T4 and T6.

In phage T4 it appears that the two phenomena of intraspecific and interspecific competition are not only conceptually joined as a strategies of resource acquisition, but their genetic determinants are physically linked and possibly co-regulated on the same section of DNA. The overlapping genes themselves are an example of resource conservation (shared base pairs) but the potential for an intricately choreographed regulatory network within this cluster of genes is far more significant. Taken together this group of genes may represent a coadaptive gene cluster - the competition cluster (Fig. 3-7). A coadaptive gene cluster is a group of tightly linked genes whose products function cooperatively in determining the

fitness of the organism (Stahl and Murray, 1966). However, as discussed next, a price is paid for such fierce competitiveness when the primary infecting phage carries damaged DNA.

The Genetic Exclusion Functions Control Phage Mating

Certain types of DNA damage involving both strands at the same position, such as double strand crosslinks, double strand breaks and gaps opposite pyrimidine dimers, can theoretically only be repaired by a recombinational process (reviewed by Bernstein, C., 1981). Recombinational repair requires exchange between two homologus chromosomes. (Recombinational repair requires the presence of redundant DNA homologous to the damaged region to serve as the source of undamaged DNA to replace the damaged section.) Therefore a phage with double strand damage in a singly infected cell can not undergo repair. Unrepaired double strand damage is lethal. However in a cell with two or more dissimiliarly damaged phage, recombinational repair is possible and usually results in viable progeny. Since the exclusion genes (primarily imm and sp) act as the gatekeeper to secondarily infecting phage (Table 3-9, column 5+ under superinfecting phage), they control the potential for recombinational repair. Phage T1 can reproduce vegetatively if one undamaged phage genome enters

the cell, or sexually if two or more phage genomes, damaged or not, infect the cell.

Bernstein et al. (1981, 1985a,b) have proposed that sexual reproduction (sex) evolved as a mechanism to provide for DNA repair. Sex is regarded as having two aspects, recombination and intraspecific outcrossing. Since the exclusion genes govern phage T4 outcrossing, it seems likely that their evolution and the evolution of recombination occurred in coordination. As sexual reproduction is nearly universal, yet very costly to the species using it (Bernstein et al., 1981, 1985a,b), its adaptive benefit remains one of the major unanswered questions in evolutionary biology. Experimental results obtained by C. Bernstein (1987) and S. Abedon (1986, personal communication) show that expression of phage exclusion gene(s) is inhibited by UV radiation (a DNA damaging agent). This allows more than one phage to infect a cell and hence an opportunity for recombinational repair of the damaged primary phage genome. This lends support to the idea that the exclusion genes are important in the phage T4 strategy for coping with DNA damage through recombinational repair. These results also raise questions as to how the exclusion genes are regulated in response to DNA damaging agents.

Usually considered of marginal value to the phage, the exclusion genes, imm and sp, now can be viewed as significant contributers in a coadaptive gene cluster encoding fundamental adaptive strategies that are universal in nature.

Gene sp and gene 40 are the same gene

The results discussed in Chapter 3, Table 3-15 and Fig. 3-8, show that recombinant plasmids which have spackle activity complement a gene 40 mutant. The results in Table 3-16 show that plasmids containing the sp gene also are able to carry out marker rescue of a gene 40 phage mutant, presumably through homologous DNA recombination. The SDS-PAGE results (Fig. 3-5) indicate that a sp containing clone (pJ011) produces a protein of about 15 kDa. This makes it unlikely that multiple ORFs are located in the 367 bp T4 insert of pJ011. Finally, I showed that 3 mutants defective in gene 40 are defective in exclusion to levels similar to a known sp mutant(Table 3-17). These findings taken together strongly indicate that the sp gene and gene 40 are one in the same. I propose redesignating the gene as sp/40. This is the first instance in phage T4 where an early expressing gene has been shown to be involved in a morphogenic pathway.

Since the phage T4 genetic map was first generated about 25 years ag , it has always been a curiosity that

gene 40, a head morphogenesis gene, was located in an early region away from the rest of the late expressing capsid-related genes. An obvious explanation now exists. It appears that spackle function took precedence in the evolution of the placement of gene sp/40 in the genome.

The intriguing sp/40 gene product

Emrick (1968) first hypothesized that the function of gp sp is primarily to maintain the integrity of the cellular envelope after infection. Since then, it has been implicated in numerous other phenotypes (reviewed in Chapter 1). Most recently, I have shown sp to be involved in head morphogenesis under the name of gene 40.

The anti-lysozyme function of sp/40 accounts for its role in genetic exclusion and prevention of both LFW and lysis from within. Since lysis inhibition itself is not understood, the function of sp gp in lysis inhibition is unclear. Gene 40 (now sp/40) is essential at high temperature and is thought to provide a bacterial inner membrane binding site for the first gp (20) in preprohead assembly. It would be unreasonable to explain gp sp/40's role in head morphogenesis by an anti-lysozyme function. This would imply a functional domain of gp sp/40 different from that which has the anti-lysozyme function. The results in Table 3-18 also argue for a multiple domain sp/40 gp. It is known that gene 40 mutations are lethal at

high temperature while these results clearly show that the sp- mutant is viable at 43°C. The sp- mutation in gene sp/40 therefore affects the sp phenotype but not the gene 40 phenotype. This suggests that there are two separate domains in gp sp/40. It is possible that there is a temperature induced conformational rearrangement of gp sp/40 that accounts for the two functional domains. In Fig. 3-4 the curve showing the exclusion of phage T4 by the sp recombinant plasmid (pJO11) has a sigmoidal shape. Gp sp expressed from the plasmid excludes T4 very efficiently at low temperatures but between 28°C and 37°C it rapidly loses its exclusion ability. However, gp 40 becomes essential at the higher temperatures. These observations are consistent with the idea that the sp/40 protein's conformation shifts with temperature.

On the other hand, Emrich (1968) reported that a sp mutant gave r-like plaques at high temperature, while rII+ plaques are normal. (r-like plaque are caused by premature lysis of the cell due to the lack of lysis inhibition by secondary phage.) This implies that the gp sp is still sufficiently functional at high temperature in wildtype infections to participate in lysis inhibition. So it appears that there is a reduced but significant sp activity even at high temperature. There is also partial exclusion of wild-type phage by the sp containing plasmid even at

high temperature. At 43°C there is about 32% exclusion by the recombinant plasmid as compared to the 95% that occurs at 22°C (Table 3-10, Fig. 3-6). Conversely, in a gene 40 mutant infection at lower temperatures phage production is decreased by as much as 80% without gp 40 (Hsaio and Black, 1978b) implying that there is still gp 40 function present. These results agree nicely with the concept of a protein existing in two conformational states over the range from 22° to 43°C but with one favored at the low temperature (sp) and the other favored at the high temperature (40). I envision gp sp/40's function in head morphogenesis as providing a stable binding site for gp 20 by associating with the bacterial cytoplasmic membrane and that this function is critical at high temperature. It may even anchor to the cell wall to stabilize itself within the fluid inner membrane. The interaction with gp 20 could possibly stabilize the gp sp/40 conformation.

As an interesting aside, e (lysozyme) mutants are only viable with an accompanying sp mutation. This viability may be due to the ability of gp 5 (lysozyme) to substitute for gene e lysozyme. This implies that gp sp inactivates gp 5. Gp 5 has been shown to have a general N-acetylmuramidase activity with no other observed site recognition requirements (Nakagawa et al., 1985). As it is unreasonable to expect that gp sp could mask many

substrate binding site in the peptidoglycan layer to inhibit gp 5 action, it seems likely that gp sp interacts directly with the gp 5 expressed by the infecting phage to prevent premature lysis from within in a sp+ infection.

A Possible New Gene Is Found

When recombinant plasmid pPP1 was tested for complementation against a gene 40- mutant a hazy spot appeared (Fig. 3-8, spot _). This is taken as a sign of a low level of complementation. The quantitative complementation assays done with the same recombinant plasmid as in the spot test indicated again a low level of complementation, about 3.4 fold over background (Table 3-15). The quantitative marker rescue assay values obtained with this plasmid indicate no cross reactivation of ocL84(40) (Table 3-16). Together these results indicate that the T4 insert in plasmid pPP1 complements but does not share homologous DNA with ocL84(gene 40), the tester phage. The insert in pPP1 is located between 23.153 and 21.195 on the T4 restriction map (Fig. 3-7) (Kutter and Ruger, 1985). Within this region there are parts of two well defined genes: gene uvsX extending from 22.033 to 23.203 kbp (Fujisawa et al., 1985) and Bgt extending from 23.815 to 21.900 kbp (Tomaschewski et al., 1985) on the phage T4 restriction map. Both of the genes have been accurately sequenced. The area between them totaling 612 bp was

previously thought to be genetically silent. It now appears that this region encodes a gene that is a suppressor of the gene forty portion of the phenotype of the sp/40 gene. Accordingly, we have tentatively designated it "sof". Although the only phenotype so far found is the suppression characteristic, by analogy with the dual function of sp it could be a third genetic exclusion gene, contributing the remaining 10 - 20% of exclusion not accounted for by imm and sp. Alternately it may just be an accessory protein aiding in the complexing of gps 40 and 20 in the initiation of prohead assembly without an additional function.

In closing, this work has provided new insights into the conceptual and functional workings of genetic exclusion in phage T4. The genetically demonstrated overlap between genes imm and 42 may have significant regulatory implications. A unique perspective linking molecular genetics to the ecological concept of competition has been presented. The functions of gps imm and sp/40 have been clarified allowing the formulation of a more accurate molecular model of genetic exclusion. By showing that genes sp and 40 are the same, an early expressing gene has been linked to a morphogenic pathway. A hypothesis involving a temperature induced conformational rearrangement has been advanced to account for the

pleiotropic effects of gene sp/40. And, speculations on the evolution of the genomic placement of gene sp/40 and the significance of genetic exclusion in the evolution of sex have been offered. Although once considered inconsequential, the cluster of genes which include sp, imm, Bgt, and 42, the competition cluster, can now be thought of as encoding fundamental adaptive stategies that are universal in nature.

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